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THE PHENOMENA OF ANURAN METAMORPHOSIS. II

(Two figures)

WILLIAM ETKIN
University of Chicago

E. OXYGEN CONSUMPTION DURING NORMAL METAMORPHOSIS

SINCE the classic demonstration of Gudernatch (1912) that thyroid feeding will precipitate metamorphic phenomena in the tadpole, the study of amphibian metamorphosis has furnished the field for some of the most brilliant and fruitful researches in endocrine physiology. The belief that has emerged from these researches may be briefly characterized as follows. The anterior lobe of the hypophysis produces a hormone which stimulates the thyroid gland in a way eventuating in the release of large quantities of the thyroid hormone. It is this thyroid hormone which is the primary stimulant, precipitating metamorphosis. Though this is but a crude statement, it will perhaps serve to make plain the intention of the present study.

Before much progress could be made in the further analysis of these relations it seemed to the author that a careful quantitative investigation of the nature of the metamorphic changes would have to be undertaken. The first published results dealt with the externally visible events; the rate of growth of the hind legs and body weight and the loss of water (1932).

On the basis of this study metamorphosis was divided for descriptive purposes into a prometamorphic period characterized by rapid hind-leg growth, followed by the metamorphic climax beginning

about the time of the emergence of the forelegs and during which tail resorption and modification of the mouth were the most obvious changes. This terminology will be found useful below. To this study may be added an earlier description of the development of the thyroid gland (1931). As these studies have served to clarify the nature of the changes and to give many suggestions as to their etiology, a further investigation of the changes in normal metamorphosis seemed desirable. Of the many questions that remain, one of the most interesting is the rate of metabolism of the animal undergoing metamorphosis.

It is commonly held that the thyroid secretion raises the basal metabolism of the cells, acting, as someone has expressed it, as a draft on a fire. Since, as stated above, we have every reason to believe that the thyroid hormone is the primary activator of the visible metamorphic phenomena, we would expect that some idea of the degree of activity of the thyroid in the secretion of its hormone during the various stages of metamorphosis could be estimated from the basal metabolism of the animal. The commonest measure of this metabolism being the rate of oxygen consumption in the non-working animal, the question of the respiratory metabolism during metamorphosis is at once raised.

In a previous paper I have had occasion to point out the paucity of literature on the descriptive phases of metamorphosis. The study of oxygen consumption is not exceptional in this regard. To my knowledge there is no report in the literature devoted to this subject, though a number of works touch upon it. The data of Gayda (1921*a*) on heat production and those of Groebbels (1922) on oxygen consumption show no effect of the inception of metamorphosis (climax phenomena) upon the metabolic rate per gram of tadpole. In both these cases, however, this point was only incidental to other purposes in the work and the individuals of the groups used were not uniform in the degree of metamorphosis that they presented, so that it is difficult to say how far this evidence can be safely interpreted. Abelin and Scheinfinkel (1923) find a drop in the total CO_2 production in one group used as control in an experimental investigation. A curve for the O_2 consumption of one individual is given by Drastich (1925). This shows a rise in early metamorphosis (metamorphic

climax) and a subsequent drop for both total and per gram respiratory rates.

It is clear that these studies are not sufficiently extensive individually or consistent collectively to permit of any conclusion. A reinvestigation of the problem covering a sufficiently large number of animals, studied under standard conditions and at known stages of metamorphosis, is plainly required.

MATERIALS AND METHODS

Specimens of the bullfrog tadpoles, *Rana catesbeiana*, obtained from an aquarium supply-house in Chicago, were used. According to the company, these animals had been collected by seining operations in Louisiana.

The animals were kept in 10 inch×8 inch glass aquaria, four animals to the aquarium. The food consisted of algae growing in the water and also cooked, dried, and ground liver which was added abundantly every 2 or 3 days. The water was changed before feeding and the aquaria placed so as to receive several hours of sunlight per day.

The oxygen determinations were made by the Winkler titration method. The apparatus used was of the continuous flow type essentially like that described by Keys (1930), somewhat simplified. The city water supply at Chicago was used. It was first aerated in a spray device and slightly supersaturated with oxygen. The water was then led off and into the animal chambers of which twelve were arranged in a row. These chambers consisted of $7\frac{1}{2}$-inch lengths of $1\frac{3}{8}$-inch glass tubing. The water flowed through these and out at the opposite ends to run into the sample bottles. Two of the chambers were reserved as blanks, and into each of the other ten an animal could be introduced. It is clear that as the water flows through these chambers the animals remove a certain amount of oxygen from it. By the Winkler titration method the oxygen content of the water coming through the empty control tubes and that through each animal chamber is measured, and from this and the rate of flow the rate of O_2 consumption for the animal could be determined. The determinations were therefore made for ten individuals at a time, one such series being run each evening.

It was important to regulate accurately the rate of flow, for that determined the oxygen tension of the water in which the tadpoles were living. This was done by drawing out capillary tubing to the proper fineness, introducing these at the entrance to the sample bottles, and also by regulating the height of the aerating tank above the water bath. In this way the oxygen tension of the water from the animal chambers was kept at about 30–40 per cent saturation. In this range it has been shown for many vertebrates and for tadpoles in particular by Helff and Stubblefield (1931) that the rate of oxygen consumption is independent of the oxygen tension. In a number of cases, however, the regulation of the rate of flow was not adequate and the oxygen tension of the water leaving the animal chambers fell well below 30 per cent saturation. In such cases it was often found that the oxygen consumption fell markedly often to less than one-half previous values. Such results had, of course, to be eliminated in considering the rates at different stages of metamorphosis; but it is of some interest to notice that these data confirm in a general way the results of Helff mentioned above. It is thus clear, however, that by maintaining a saturation of about 40 per cent complications due to differences in oxygen tension are avoided.

In previous work I found that there is a marked drop in metabolism from day to night. This appeared to be correlated with the reduced activity of the animals in the dark. To take advantage of this all determinations were made at night and at approximately the same hours. The animal chambers were further screened off from the room lights. Under such conditions of reduced illumination it was found that the animals lay almost perfectly quiet for long periods of time. I have repeatedly watched the set-up of ten animals for 10–15 minutes at a time and in all have seen only a few isolated tail movements. It is clear, then, that the activity of the animal is not an important factor in the results and we are dealing here with a rate of metabolism which approximates the so-called "basal metabolism" as closely as is generally available.

Preliminary work had also shown that about an hour is required after the introduction of the animal into the chamber before an equilibrium is reached between the oxygen tension of the input and outflow waters. This is due to the fact that there are about 100 cc.

of water in the tube which must be reduced in its oxygen tension to the equilibrium point. In all experiments, therefore, the animals were permitted to remain quiet and undisturbed in the tube for 3–4 hours before a reading was taken. No attempt was made to starve the animals before introduction as it was found that 24 hours' starvation did not have much effect on the intestinal contents and interfered with the growth of the animal.

Each of the four animals of an aquarium were separately identified by small cuts in the tail fin so that individual records could be kept. In general, determinations of oxygen consumption were made every 3–4 days during the pre- and prometamorphic period and daily reading during earlier climax. The readings could be continued only until the time when the tail was one-half to two-thirds resorbed. Until this time the animals are primarily water-breathers gulping air into the lungs only occasionally, and are capable of living without access to air. However, after a definite period (when the tail is about one-half resorbed) the animals change their mode of respiration so that they will breathe only air and will not pump water through the gill chamber. At this time it is impossible to use the present apparatus, for the animals drown in the tubes, and the readings were discontinued at this point. However, for answering the questions raised, I believe the period tested to be adequate since it covers the period from before metamorphosis until the time the climax phenomena are more than one-half complete. Any effects of the factors inducing metamorphosis should therefore be apparent.

Record was taken of the body weight and of hind-leg length at weekly intervals in earlier stages and daily body-weight records were made during the climax.

In all, forty animals were investigated. For a large number, however, the records are not complete enough to permit their being used in connection with the present discussion. This is partly due to the limitation of time, partly to the loss of animals by accident during the long series of readings. As a result, the records of only twenty-four animals are sufficiently complete to cover either of the two critical stages discussed below. It will be seen, however, in the discussion of the results that the principal points can be sufficiently established from these records.

Before the results in relation to metamorphosis can be discussed it is necessary to consider the accuracy of the technique. As mentioned above, in each set-up, two control samples were taken. These agreed very well. If the two samples are averaged and the deviation of the samples from the average expressed as per cent of the average, then this population of deviations showed a mean of 0.3 per cent and a S.D. of 0.26 per cent. The repeatability of the technique is, therefore, more than adequate for the present purpose. The fact that there is a continuous flow of water through the chambers eliminates the criticism that accumulation of organic wastes interferes with the titration. Three tests for the titration of I_2 introduced into the water from the animal chamber as compared with aliquot amounts introduced into distilled water showed practically no loss. It would seem, therefore, that the Winkler titration method may be used satisfactorily in this procedure.[1]

A point of considerable interest is the variation of the readings from hour to hour as the animals lay in the chamber. This was determined by making a second determination $1\frac{1}{2}$ hours after the usual one in forty-nine cases. The two determinations were then treated as the duplicate blank samples mentioned above. This population of deviations showed a mean of 4.0 per cent and a S.D. of 4.2 per cent. We have in this, then, a measure of how accurately our reading at a given time represents the true average reading for that period.

RESULTS

The numerical results for the twenty-four specimens which are suitable for our discussion are summarized in Table I for oxygen consumption and Table II for body weight.

The main tendencies of these results can best be brought out by combining the individual records. This was done by plotting the individual curves upon semilogarithmic graph paper. In this way a graph is constructed in which the slope of the line indicates the proportionate change independently of the absolute values involved.

[1] Recent work in this laboratory under Dr. Allee's direction has revealed in certain waters, and under conditions approaching those used in these experiments, a considerable error in the Winkler titration resulting from the presence of nitrites. An analysis of the water as used in the present investigation was kindly made by Dr. Schuett; this showed no appreciable amounts of nitrites.

TABLE I

SHOWING THE OXYGEN CONSUMPTION OF TADPOLES

DATE	SPECIMEN NUMBER SERIES 2BR																						
	2	3	4	6	7	8	11	12	13	15	16	18	20	23	25	26	33	34	35	36	37	38	40	42
May 20	.....	.....	.....	.....	.....	.....	2,906	.....	2,733	.....	.....	3,125	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
21	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
22	2,921	3,001	.....	.....	.....	.....	.....	.....	.....	.....	0,972	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
23	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,588	.....	0,773	2,053	1,861	.....	1,078	.....	.....	.....	.....
24	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,982	2,049	.....
25	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
26	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
27	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
28	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
29	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
30	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
31	3,042*	2,752	.....	2,715	.....	2,966	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
June 1	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,760	0,832	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
2	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,192	.....	.....	2,220	1,760	.....	1,620	.....	1,730	1,750	.....
3	.....	2,724	3,210	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
4	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,141	2,159	.....	.....	1,753	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	2,153	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,503	1,700	1,574	1,598	2,108	1,583	1,902	.....
6	1,841	2,767	2,828	.....	2,727	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
7	1,899	.....	.....	2,834	.....	.....	2,718	3,174	3,025	2,180	1,237	.....	2,862	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
8	.....	.....	2,816	.....	.....	.....	2,477	.....	2,736*	.....	.....	.....	.....	1,558	2,474	0,886	1,998	2,338	.....	.....	.....	.....	.....	.....
9	.....	.....	3,652	.....	.....	.....	.....	.....	2,476	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,058	1,519	2,197	1,812	2,215	.....
10	.....	.....	1,703*	3,108	3,031	2,707	2,557	2,732	2,086	1,408†	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
11	.....	.....	2,279	.....	.....	.....	3,058	.....	1,852	.....	1,676	3,661	.....	2,056	3,056	.....	.....	.....	.....	.....	.....	.....	.....	.....
12	.....	3,814	2,036	.....	2,941	2,818	3,238	2,881	1,982	2,385	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
13	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,456	.....	2,559	2,423	2,158	1,866	2,725	2,010	2,492	.....
14	.....	3,622	2,292	3,042*	3,268	3,227	2,976	2,810	2,275	2,806	.....	.....	†	.....	.....	.....	.....	†	.....	.....	.....	.....	.....	.....
15	.....	.....	.....	.....	.....	.....	*	.....	.....	.....	1,475	3,324	2,901	2,341	3,020	.....	3,342	.....	.....	.....	.....	.....	.....	.....
16	.....	2,752*	.....	2,532	*	.....	2,886	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,365	2,195	2,502	3,211	2,490	2,805	.....
17	.....	2,777	2,351	2,378	3,608	3,330*	2,772	3,338	.....	3,027	1,760	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
18	.....	3,259	.....	2,718	2,731	3,037	3,209	3,847	.....	.....	.....	3,182	3,272	.....	.....	.....	†	.....	.....	.....	.....	.....	.....	.....
19	.....	3,064	.....	3,100	2,684	2,680	2,682	.....	.....	.....	.....	.....	.....	.....	2,789	.....	2,470	2,253	2,483	.....	†	.....	.....	.....
20	.....	2,848	.....	2,824	2,531	2,381	2,641	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,643	2,497	2,722	2,887	.....
21	.....	2,807	.....	.....	2,397	2,663	2,575	3,033	.....	2,698	1,804	3,253	.....	.....	.....	.....	.....	.....	.....	.....	.....	†	.....	.....
22	.....	2,427	.....	.....	2,279	2,670	2,336	.....	.....	.....	.....	*	3,070	2,240	2,851	.....	.....	.....	.....	.....	.....	.....	.....	.....
23	.....	.....	.....	.....	.....	2,316	.....	3,144	.....	.....	.....	2,756	.....	.....	.....	.....	2,570	2,418	2,045	2,203	2,262	2,363	.....	.....

The day the hind legs reached 10 mm. in length is indicated by † and the day on which the forelegs emerged is indicated by *. The figures are given in terms of cubic centimeters of oxygen (at 20° C. and 760 mm. pressure) consumed per animal per minute. They have, however, been multiplied by 10^6 in order to eliminate the decimal point.

TABLE I—*Continued*

Date	Specimen Number Series 2BR																							
	2	3	4	6	7	8	11	12	13	15	16	18	20	23	25	26	33	34	35	36	37	38	40	42
June 24	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
25	.....	.....	.....	.....	.....	.....	.....	3,625*	.....	3,502	1,886	2,549	3,240	2,250	2,516	1,191	.....	.....	.....	.....	.....	.....	.....	.....
26	.....	.....	.....	.....	.....	.....	.....	3,429	.....	.....	.....	2,671	.....	.....	2,420*	.....	2,600	2,648	2,596	1,772	.....	2,013	2,414	.....
27	.....	.....	.....	.....	.....	.....	.....	3,016	.....	.....	2,258†	2,504	2,987	.....	2,287	.....	.....	.....	.....	.....	.....	.....	†	1,301
28	.....	.....	.....	.....	.....	.....	.....	2,520	.....	.....	.....	2,524	.....	.....	2,289	1,090	3,078	3,332	2,921	2,456	2,572	.....	.....	.....
								2,689			.....	2,422	.....	2,102	2,140	1,114	2,851	2,312	2,622	1,790†	2,234	.....	.....	.....
29	.....	.....	.....	.....	.....	.....	.....	3,145	.....	.....	.....	2,709	.....	.....	2,143	.....	.....	.....	.....	.....	.....	2,632	2,865	2,130
								3,130			.....	2,497	.....	.....	2,029	.....	.....	.....	.....	.....	.....	2,218	2,875	2,130
30	.....	.....	.....	.....	.....	.....	.....	2,646	.....	.....	1,864	.....	3,062	2,534	2,219	1,512	2,566	2,789	.....	.....	.....	.....	.....	.....
July 1	.....	.....	.....	.....	.....	.....	.....	2,579	.....	.....	.....	.....	.....	†	2,236	.....	.....	.....	3,056	2,534	2,673	2,584	2,925	2,525†
2	.....	.....	.....	.....	.....	.....	.....	2,348	.....	.....	2,945	.....	3,308	2,706	.....	1,576	3,427	3,122	.....	.....	.....	.....	.....	.....
3	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
4	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,233	.....	3,293	2,321	.....	1,533	3,324	3,091	.....	2,314	2,812	3,010	2,707	.....
6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
7	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	3,020	2,682	.....	1,161	3,304	2,892	.....	2,041	1,867	2,323	2,390	.....
8	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,650	.....	3,144	.....	.....	1,411	2,892	2,998	.....	.....	2,356	.....	.....	2,553
9	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,784*	2,789	.....	.....	3,324	2,867	.....	2,619	2,503	2,480	2,776	2,694
11	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
12	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,848	.....	.....	.....	.....	.....	3,521	2,284	.....	2,574	2,683	2,550	.....	2,828
13	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,752	.....	2,709	2,954	.....	1,456	2,773	2,152	.....	.....	2,729	2,604	2,433	.....
											2,570	.....	2,945	2,923	.....	1,421	3,538	2,185*	.....	.....	2,858	2,859	2,934	.....
14	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,404	.....	.....	.....	3,470	2,581	.....	3,184	2,646	2,768	3,048	2,862
													2,467	.....	.....	.....	3,581	3,017	.....	2,970	2,470	2,795*	3,057	2,581
15	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,480	.....	.....	2,362	.....	1,574	3,114*	2,196	.....	2,301	2,368*	2,427	2,457	.....
16	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,131	.....	2,995	.....	2,385	.....	2,269
17	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,577	.....	1,756	.....	1,698	.....	2,552	.....	2,015	.....	2,501
18	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,700	.....	.....	.....	2,996	.....	2,097	.....	2,246*
19	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1,451	.....	.....	.....	3,257	.....	.....	.....	2,613
20	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,557	.....	.....	.....	2,030
21	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,290	.....	.....	.....	2,130
22	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1319†	.....	.....	.....	2,335	.....	.....	.....	1,788

TABLE II

Body Weights of Metamorphosing Tadpoles

Date	Animal Number																								
	2	3	4	6	7	8	11	12	13	15	16	18	20	23	25	26	33	34	35	36	37	38	40	42
	Body Weight in Grams																							
May 22	11.8	11.1	13.0	13.0	11.6	12.5	9.7	10.2	10.6	8.0	6.4	10.0	7.4	5.5	9.9	3.0	9.1	8.7	5.6	6.6	9.2	7.8	9.4	....
June 1	10.3	11.2	13.4	12.6	10.6	12.8	11.8	12.0	12.8	8.2	6.8	12.2	6.4	6.0	10.6	3.0	9.4	9.6	5.6	6.9	8.1	7.5	8.8	....
5	8.2	10.1	13.3	12.9	10.9	13.1	10.2	10.7	11.2	7.6	6.2	11.2	6.1	5.6	9.6	....	9.2	8.8	6.4	7.0	8.6	7.7	8.9	....
8	7.0	9.7	11.6	12.3	10.9	12.8	11.1	11.4	11.9	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
12	6.5	10.5	10.4	12.5	9.5	12.1	9.5	11.1	9.5	8.1	6.5	13.0	7.3	5.6	10.7	2.8	10.0	9.3	6.6	....	....	....	9.2	....
14	....	10.4	10.0	11.7	9.3	12.0	9.7	....	8.5	....	....	....	....	....	....	....	....	9.0	....	....	....	....	....	....
19	....	8.6	....	9.5	7.8	10.4	8.0	12.3	....	10.0	7.3	13.2	8.9	6.1	11.9	....	11.2	10.0	7.9	8.5	11.0	9.1	10.3	....
21	....	....	....	....	6.7	9.0	7.1	12.1	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
26	....	....	....	....	5.2	6.2	5.5	10.6	....	10.9	6.6	9.8	10.1	6.6	10.3	4.0	12.6	11.2	9.6	9.2	11.8	10.0	11.4	....
27	....	....	....	....	....	....	....	10.3	....	....	....	9.5	....	....	9.6	....	....	....	....	....	....	....	....	....
29	....	....	....	....	....	....	....	9.8	....	....	....	8.5	....	....	8.2	....	....	....	....	....	....	....	....	....
July 1	....	....	....	....	....	....	....	9.1	....	....	....	....	....	....	7.9	....	....	....	....	....	....	....	....	....
3	....	....	....	....	....	....	....	8.4	....	....	7.4	10.2	10.2	7.8	7.1	4.6	12.0	10.9	9.9	9.6	11.3	9.8	10.8	7.4
7	....	....	....	....	....	....	....	....	....	....	....	....	9.7	....	....	....	....	....	....	....	....	....	....	....
10	....	....	....	....	....	....	....	....	....	....	7.6	....	....	8.2	....	5.3	13.0	11.3	....	10.5	11.4	9.8	11.8	8.1
13	....	....	....	....	....	....	....	....	....	....	....	8.1	....	....	....	....	11.8	9.3	....	....	10.2	8.8	....	....
15	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	11.3	8.4	....	....	9.8	8.0	....	....
17	....	....	....	....	....	....	....	....	....	....	....	....	6.2	8.7	....	6.3	....	7.0	....	10.8	....	7.4	....	7.5
20	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	9.8	....	....	....	5.7
22	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	5.6	....	....	....	9.3	....	....	....	5.1

In this form, therefore, the graphs can be superimposed without regard to the differences in absolute values. The upper sets of points in Figures 1 and 2 show the results obtained by superimposing the points according to the following procedures.

For each graph the best straight-line fit determined by inspection was drawn. A piece of thin celluloid sheet was then placed over the graph. This was adjusted so that the X-axis was exactly parallel to a line drawn on the celluloid. The value of the curve for the day the hind leg reached 10 mm. was adjusted to be just beneath a point marked on the celluloid which we shall call the "fulcrum." The points of the graph desired were then marked on the celluloid. This was done on the same piece of celluloid for each of the thirteen specimens which covered the period satisfactorily from before prometamorphosis to the day before emergence. Figure 1 shows the resulting distribution of the points.

Another similar procedure was followed using the value of the curve at two days before emergence of the forelegs as a fulcrum and covering the points in prometamorphosis and the metamorphic climax, until the last available day, i.e., the time the tail is about one-half to two-thirds resorbed. Figure 2 shows the results of this procedure.

Each graph gives us a picture of the changes in oxygen consumption with respect to some definite period in the metamorphic process. By setting the level of the fulcrum at 100, we can, by simply setting down the proper logarithmic intervals, represent the percentage deviation of any point from this value.

The period of prometamorphosis.—From Figure 1 we can see that there is an increasing oxygen consumption as the development of the animal proceeds from considerably before the onset of prometamorphosis to the day of emergence of the forelegs. No specific change appears with the onset of prometamorphosis (i.e., H.L.L. = 10 mm.).

Since our primary interest is with the rate of metabolism of each unit of tissue, we must take the growth of the tadpole into consideration. For showing the general tendencies of the body growth in weight, the same procedure of plotting the values on semilogarithmic paper and superimposing the points seemed most advantageous. The lower parts of Figures 1 and 2 show the results, using the same

specimens and the same points in metamorphosis as fulcra as in the upper curves.

We are immediately struck by the similarity of the curves for total metabolism and for body weight. Not only are they similar in form, but the percentage deviation from the fulcrum-point level is apparently much the same. In short, as the body weight increases the total metabolism increases in direct ratio so that it is clear that

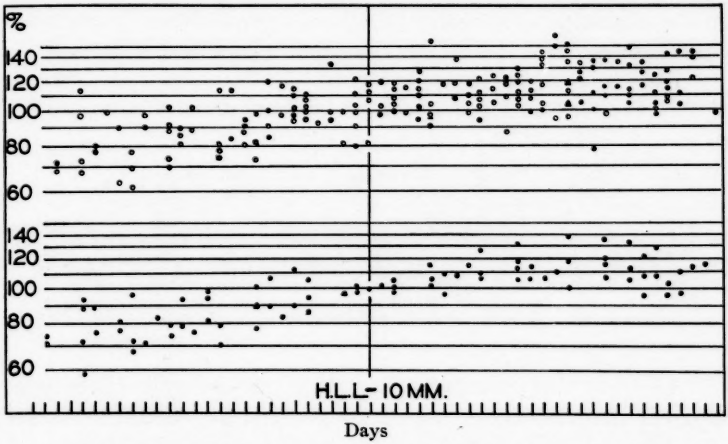

FIG. 1.—Shows the distribution of points resulting from the superposition of individual curves for thirteen animals. The day the hind legs reach 10 mm. is used as fulcrum. Upper curve for O_2 consumption and lower for body weight. Open circles indicate one reading, filled-in circles indicate two readings at the same spot, triangles indicate three readings, and a large square indicates four readings.

the *rate of respiration per unit weight remains approximately constant throughout the period studied.* This holds until a few days before emergence of the forelegs, when a drop in weight complicates the situation. The divergence at this time will be discussed later.

It is not satisfactory, however, to leave the analysis at this point, for I have previously shown (1932) that the growth phenomena of the tadpole at this time are complicated by a relative loss of water, so that the rate of growth of the animal in dry weight is greater than the total weight growth rate. These results were confirmed for the present batch of animals by determining the water content of the

animals left when it became necessary to discontinue the determinations. The figures obtained are shown in Table III.

If these results are compared with my previously published figures, a good agreement is readily seen. I think we are therefore justified in applying the general concept arrived at in this previous study to the present group of animals by sayings that there is a gradual relative loss of water during growth previous to prometamorphosis and an acceleration of this loss during prometamorphosis. In the climax an exceedingly rapid desiccation of the tissues takes place.

TABLE III

DESICCATION PHENOMENA IN METAMORPHOSIS

An. No. Series 2BD	Description	Per Cent $\frac{\text{Dry Weight}}{\text{Wet Weight}}$
5	Early prometamorphosis	11.9
3	Mid-prometamorphosis	11.3
6	One day before emergence	14.9
2	Mid-climax	16.8
4	Mid-climax	15.6
1	Complete metamorphosis	20.3

If we apply this concept to the data given above, it will become apparent that the rate of respiration per unit dry weight decreases as the animal grows larger and the rate of decrease is accelerated during prometamorphosis. The amount by which this rate is decreased during prometamorphosis may be estimated at 30 per cent since that is approximately the difference in the amount of dry weight represented by a unit of total weight at the beginning and end of prometamorphosis.

We may summarize our consideration of this period as follows:

1. As the tadpole develops the rate of oxygen consumption rises, the rate per unit of total weight remains approximately constant, and the rate per unit of dry weight decreases.

2. In comparing pre- with prometamorphosis we find that the rate of increase of the total oxygen consumption and the rate of metabolism per unit of total weight remain unchanged and unaffected by prometamorphosis and the rate per unit of dry weight is decreased faster in prometamorphosis than previously.

Metamorphic climax.—For a similar consideration of the changes occurring at the beginning of the metamorphic climax, Figure 2 will serve. It is based upon curves for fifteen animals, only four of which are also to be found in the previous group. Here we notice that the period of prometamorphosis represented to the left of the arrow shows the same characteristics as displayed by this period in Figure 1. At about the day of emergence, however, we notice there is a

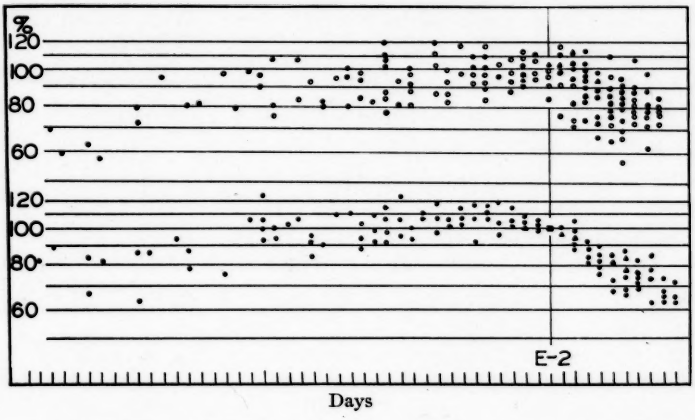

FIG. 2.—Shows the distribution of the points resulting from the superposition of individual curves for fifteen animals. The fulcrum is set at 2 days before the emergence of the forelegs. Arrangement as in Figure 1.

sharp drop in the total oxygen consumption, which continues as long as the present experiment could be run (up to 7 days after emergence). If we consider the body weight, again we are struck by a decided agreement. The drop in body weight begins somewhat before the drop in oxygen consumption, but otherwise the curves are almost identical even in the approximate percentage of the drop. It is clear, therefore, that within the variation of the figures there is a fairly constant rate of metabolism per unit body weight through prometamorphosis and early climax, with the exception that there is a slight rise in this rate as a result of the earlier falling-off of the body weight while the total oxygen consumption remains high.

Again if we consider the changes in water content, I have previ-

ously found that the period of metamorphic climax, the first one-half of which is represented in our figure, is characterized by a very rapid desiccation. It is clear, therefore, that if the rate of respiration per unit body weight remains constant, the rate per unit dry weight must fall sharply. The figures of my previous study on the rate of desiccation are not sufficiently extensive to make possible an accurate estimation of the amount of drop involved in this short time, but the fact that the tadpoles during early metamorphic climax are undergoing rapid desiccation seems perfectly clear. With regard to the slight increase coming just before the emergence of the forelegs, I am of the opinion that it has no significance in the sense of a fundamental rise in the metabolism of the animal. This appears on two grounds: (1) There is no increase in the rate of change of the total metabolism as there must be if the animal were to undergo a fundamental increase of the metabolic rate. (2) The loss of weight, which is the basis of the increased metabolic rate per unit body weight, has been shown in my previous studies to be a result partly of the loss of intestinal contents and partly to the beginning of the characteristic rapid dehydration of the metamorphic climax.

We may therefore summarize our understanding of the metabolic rate of the tadpole in relation to the early metamorphic climax as follows:

1. The rate of oxygen consumption of the animal as a whole drops more than 20 per cent during the first few days of the metamorphic climax, i.e., after emergence of the forelegs. The rate per unit of the total weight remains approximately constant, owing to the loss of body-weight coincidentally with the drop in metabolism. The rate of oxygen consumption per unit of dry weight drops markedly.

2. The rate at which the oxygen consumption per unit dry weight is decreasing is faster during early climax than previously.

Thus far our consideration of the results has been confined to the general tendencies. In a developmental process such as metamorphosis we are led to expect that the essential changes would show a high degree of constancy in the time and nature of their appearance. It is a matter of interest to know how far we can extend our conclusions to individual animals. For this purpose the available facts on

the respiratory rate and growth changes for the individual animals must be consulted. It is impractical to publish the 24 graphs available, but Table I is so arranged as to give as graphic a representation of the available data on oxygen consumption as could be obtained. When these are examined, we find that of the fifteen specimens showing a sufficient number of readings on either side of emergence (specimens 2, 3, 4, 6, 7, 8, 11, 12, 13, 18, 20, 34, 38, 42), all can be seen to show a decided drop at or soon after the day of emergence. That this drop must be real in each case can readily be seen if the relatively small variability of the successive readings on the same animal is recalled. Some variation appears in just when the drop first occurs, as in specimens 8 and 12 it is 2–3 days after emergence and in specimens 20 and 34 apparently on the day before or day of emergence of forelegs. It seems sufficiently clear, therefore, that a drop in total metabolic rate must be considered a characteristic event of early climax taking place in all normally metamorphosing animals.

The prometamorphic changes in metabolic rate, lacking as they do the striking definiteness of the change at emergence, are more difficult to follow in individual cases. The author believes, however, that an examination of Table I will reveal that in each adequate case there is a decided tendency for the results to conform to the general tendency revealed by the combined results. We are led to believe, therefore, that the oxygen consumption, like the other metamorphic events studied, displays a regular, perfectly definite change such as has been summarized above for the group as a whole.

DISCUSSION

In the introduction the expected results of a study of the metabolism of metamorphosing tadpoles was discussed. It was there pointed out that according to current theory a distinct increase in oxygen consumption was to be anticipated. The actual results found in the present study are diametrically opposed to this.

This contradiction compels us to re-examine the grounds of our reasoning. As previously stated, the direct evidence on the metabolic rates of metamorphosing amphibia is very scarce and unsatisfactory. The assumption, nevertheless, that there is a heightened

metabolism accompanying and perhaps of causal significance in the transformation of these animals is very general (thus see Jahrish, 1920; Uhlenhuth, 1921; Huxley, 1925; Schulze, 1930; Adams, Kuder, and Richards, 1932).

Uhlenhuth, for example, writes (1921, pp. 197): "From these facts it seems evident that the amphibian metamorphosis is the result of a highly increased metabolism, or, more correctly, metamorphosis seems to result if metabolism is increased in such a degree and manner that catabolism becomes higher than anabolism."

The most direct evidence brought forward for this view of metamorphosis seems to rest for the most part on several facts.

1. There is a considerable loss in weight in metamorphosis.

2. The rise in metabolism is considered the most characteristic effect of hyperthyroidism in mammals.

3. Substances presumed to increase metabolism have been reported to accelerate the metamorphosis-inducing effects of thyroid treatment and vice versa (thus Jahrish, Uhlenhuth, Huxley as above).

4. Thyroid medication is reported to accelerate the metabolic rate of larval amphibia in which metamorphosis is produced (discussion below).

A brief consideration and criticism of these points seems to me necessary for a clarification of the problem.

The loss of weight in normal metamorphosis I have previously shown (1932) to rest almost if not entirely upon the loss of intestinal contents and water. It cannot therefore be presumed a priori to be evidence for increased catabolism. The argument from the work on mammals is at best an unsafe analogy; the third point above assumes that an increase in metabolism takes place rather than proving it. The last point is, however, the most direct and seemingly the most conclusive in indicating that a rise in metabolism must at least accompany metamorphosis.

On the subject of metabolism of thyroid-medicated amphibia I am familiar with the following reports: Gayda (1921*b*) Groebbels (1922), Abelin and Scheinfinkel (1923), Helff (1926), and Belehrádek and Huxley (1927). Abelin and Scheinfinkel report an initial slight rise in total CO_2 production followed by subsequent marked fall.

The other authors mentioned have considered not the total metabolism but the metabolism per unit body weight, and have uniformly come to the conclusion that at some time in the artificial metamorphosis precipitated there is a rise in metabolism. Groebbels, Helff, and Bělehrádek and Huxley have worked on oxygen consumption while Gayda has used heat production as a measure of metabolic rate. If we use the more complete reports of Helff and Gayda and estimate from their data the total metabolism of their animals, we find that they agree with that of Abelin and Scheinfinkel in showing a decrease with the process of metamorphosis. In this respect, then, the work on thyroid medication is in essential agreement with my results on normal metamorphosis.

And yet at first sight the insistence of the more recent workers (Helff, also Bělehrádek and Huxley) upon using the rate per gram of body weight as a basis of discussion seems justified, and it is on this basis that a rise in metabolism is predicated. But a more careful consideration will, I think, throw serious doubt upon the value of this procedure. In the first place, we find that one of the most striking results of thyroid feeding is a sharp decline in body weight. From the studies of Romeis (1915) and the comparison with a similar drop in normal metamorphosis mentioned above, it is clear that a large part of the absolute weight loss is a result of dehydration and intestinal evacuation. If the conditions are comparable to normal metamorphosis (which, of course, is the assumption upon which the foregoing worked), then a unit of total weight would assume entirely different values in terms of the amount of protoplasm represented at different stages in artificial as well as in normal metamorphosis. Therefore, the use of the total weight as a measure of the amount of metabolizing tissue is seriously to be questioned. In the second place, a rising in metabolism should show up initially in the total metabolism, as indeed is the case in thyroid medication in mammals. This, however, does not appear in the reports mentioned above except that of Abelin and Scheinfinkel.

For these reasons I am of the opinion that the question of the influence of thyroid upon larval amphibia must be considered as unsettled and indeed must remain so until more is known of exactly

what the body-weight changes that result from thyroid medication involve.

It must be considered, therefore, that an accelerating effect of the thyroid treatment upon the metabolism of larval amphibia has not been demonstrated. It then becomes understandable that we should have failed in finding an increased metabolic rate in metamorphosis, for we see that the fundamental assumption upon which our expectations were based—namely, that the thyroid secretion stimulated basal metabolism—was not warranted as applied to the amphibian larva. We are therefore free to draw the natural conclusion that thyroid hormone, in quantities such as are inolved in the normal induction of metamorphosis, does not raise the metabolic rate of amphibian larvae. In this connection it is of great interest to note that Henschel and Steuber have recently shown (1931) that the adult frog does not respond to thyroid treatment by any increase in metabolic rate.

The problem of the cause of the drop in metabolism in early metamorphic climax cannot of course be solved from the available data. An important contributing factor may be pointed out, however. It is well known that a cessation of feeding results in a drop in basal metabolism. This has been found for animals generally, for example, Bělehrádek and Huxley (1927) in the axolotle. Since the tadpole in the metamorphic climax ceases to feed and quickly empties its intestine, it would appear that some part of the drop, at least, is likely due to this factor. A test of this point is difficult in the tadpole, for in ordinary starvation the intestine is very slowly emptied so that conditions comparable to those of normal metamorphosis are not attained. Other possible factors contributing to a decrease such as the change in the hydration of the tissues might be mentioned but cannot as yet be evaluated.

A point of general interest in these data on the metabolic rate of the developing tadpole may be pointed out. It has been found to be a general rule that the metabolism of an animal per unit weight decreases with age. As we have seen above, this does not appear to be the case in the bullfrog tadpole over the later part of its existence as such. When, however, the data are analyzed in terms of the rate of metabolism per unit dry weight, the tadpole is seen to fall clearly in

line with other animals investigated. We see here another example of the importance of taking into account the peculiarly rapid desiccation of the tissues that the tadpole undergoes in any consideration of the growth of the animal.

SUMMARY

An investigation of the rate of oxygen consumption of bullfrog tadpoles in relation to the various stages of metamorphosis was made. The conclusions drawn may be summarized as follows:

1. During the period of rapid hind-leg growth (prometamorphosis) the total O_2 consumption rate rises, but the rate per unit tissue remains constant. The rate per unit dry weight falls more rapidly than previously.

2. During the first part of the period characterized by the involution of larval organs (metamorphic climax) the total rate falls coincidently with the body weight. The rate of metabolism per unit dry weight falls rapidly.

A consideration of the literature further strengthens the conclusion that there is no reason at present for ascribing any causative significance in metamorphosis to the supposed metabolism-accelerating influence of the thyroid. This supposition itself is indeed not adequately supported as applied to larval or adult frogs.

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THE EFFECT OF LIGATION OF THE PULMONARY ARCH ON AMPHIBIAN METAMORPHOSIS[1]

(Eight figures)

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THE physiology of the sixth aortic (pulmonary) arch in the amphibian can be appreciated only by considering the changes that occur at the time of metamorphosis. The following description is based on the condition in *Ambystoma tigrinum*. During the larval stages gills are found in arches 3, 4, and 5; and blood passing through these arches to the gills is, under normal conditions, relatively low in oxygen content. The sixth aortic, or pulmonary, arch in larval stages has only a vestige of a gill, consisting of the anastomosis in the gill blade which separates the ventral and dorsal portion of the sixth arch (see Fig. 1). Only a relatively small amount of blood passes through this anastomosis to the pulmonary artery. Most of the blood going from the heart to the lung of a larval salamander passes, therefore, first through the gills and reaches the pulmonary artery by means of the ductus arteriosis (see Fig. 1). The ventral portion of the pulmonary, or sixth aortic, arch, as such, is practically non-functional in the larval animal.

During metamorphosis, however, the gills are reduced, and aortic arches 3, 4, and 5 necessarily relinquish the respiratory rôle, as this is taken over by the pulmonary, or sixth. The manner in which this is brought about is significant. At the beginning of metamorphosis,

[1] It affords me great pleasure at this time to be able to express my gratitude to Dr. E. Uhlenhuth for his suggestions, inspiration, and valuable criticism, and to extend my thanks to Dr. R. J. Gilmore, of Colorado Springs, for supplying the animals used in these experiments. I am also indebted to Dr. H. B. Wylie and the staff of the Biochemistry Department of the University of Maryland Medical School for supplying the large quantities of distilled water that were used in Experiment II; to Carl D. Clarke and staff of the Art Department for photographing the animals; to Dr. Frederick Fenger of Armour and Company for supplying the thyroid powder; and to Mr. R. Fox for permission to collect and separate the anterior lobe and posterior lobe in the *abattoir* of Greenwald & Company.

EXPLANATION OF PLATES

Ext. Car., external carotid	R. A., right atrium
Int. Car., internal carotid	S. V. sinus venosus
L. A., left atrium	V., ventricle

PLATE I

FIG. 1.—Illustration of the conditions found in a larva of *Ambystoma tigrinum*. Note that all aortic arches (ventral portions) are stippled to indicate that the blood carried by them is relatively low in oxygen content.

FIG. 2.—The heart and aortic arches of an adult *Ambystoma tigrinum*. Note that the anastomosis on the ventral portion of the sixth aortic arch has changed to a single large arterial trunk. This vessel is stippled to indicate that the blood carried by it is lower in oxygen content than that sent to aortic arches 3 and 4 (carotid and systemic). The cutaneous and pharyngeal branches are omitted.

PLATE II

FIG. 3.—Heart and aortic arches of *Necturus*. Note the absence of the spiral valve and ventral portion of the sixth aortic arch (indicated by broken lines - - -). The ventral portions of arches 3, 4, and 5 are stippled.

FIG. 4.—The heart and aortic arches in a ligated larva of *Ambystoma tigrinum*. Note that the condition is exactly the same as in *Necturus* except that a spiral valve is present in the ligated animal.

PLATE I

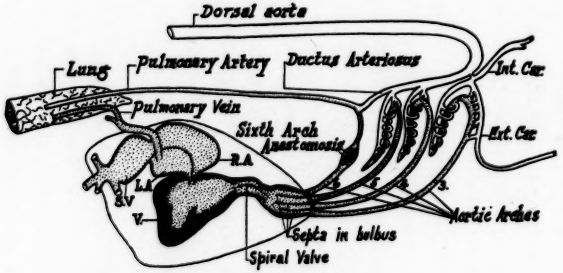


Fig. 1. Diagrammatic Side View of Heart and Aortic Arches in A. tigrinum Larva.

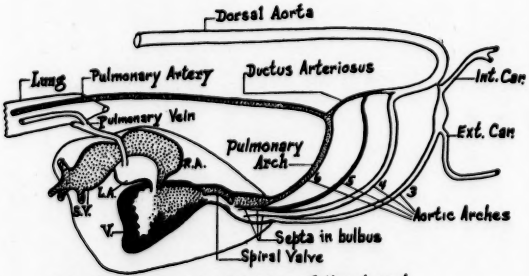


Fig. 2. Diagrammatic Side View of Heart and Aortic Arches in A. tigrinum. ADULT.

PLATE II

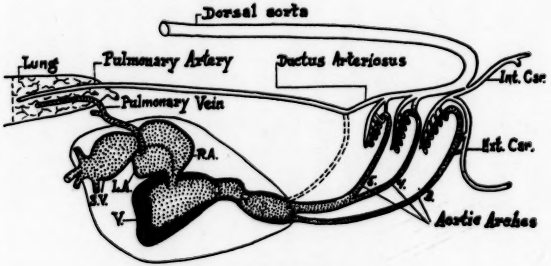


Fig. 3. Diagrammatic Side View of Heart and Aortic Arches in Necturus

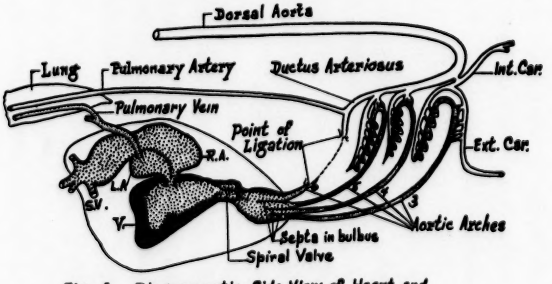


Fig 4. Diagrammatic Side View of Heart and Aortic Arches in Operated A. tigrinum.

and as this progresses, there is a steady increase in the quantity of blood that passes directly from the heart to the lung and skin through the pulmonary-arch anastomosis. Soon the smaller anastomotic branches are obliterated and a single vessel remains (see Figs. 1 and 2). This becomes the pulmonary artery and, in the adult, is large enough to carry a considerable quantity of blood to regions where it may be oxygenated (the skin, pharynx, and lungs). The oxygenated blood necessarily returns from these regions to the heart.

Blood entering the larval heart is, for the most part, low in oxygen content. As the anastomosis of the sixth aortic arch becomes a large arterial trunk, more and more blood is sent to the lungs, skin, and pharynx to be oxygenated before it enters the heart. It necessarily follows that there is a corresponding gradual increase in the oxygen content of the blood flowing from the heart through the gills (aortic arches 3, 4, and 5). It was, therefore, suspected that the increase in oxygen content of the blood sent to the gills might be one of the factors involved in gill reduction. If that were true, the ventral portion of the sixth, or pulmonary, arch and spiral valve would be an indispensable mechanism in amphibian metamorphosis.

This hypothesis received considerable support by the condition found in *Necturus*, a perennibranchiate form. In previous papers (Figge, 1928, 1929, and 1930) it was pointed out that in *Necturus* the ventral portion of the sixth aortic arch (together with the spiral valve) is lacking. Evidentally in *Necturus* the mechanism to shift the respiratory function from the gills to the lungs, skin, and pharynx is absent. It was thought that this anatomical condition accounts for the persistence of the gills in *Necturus* (see Fig. 3).

This hypothesis was tested in the larval *Ambystoma tigrinum* of Colorado (Colorado axolotl), as described in a previous paper (Figge, 1930), by ligating the sixth aortic arch. It was found that, in Colorado, ligation of the sixth aortic arch inhibits metamorphosis. Moreover, it could be shown that injection of a small amount of thyroid substance, while it produced metamorphosis of normal animals, was not sufficient stimulus to reduce the gills of ligated animals, although it led to a profuse skin-shedding and eventual death of the ligated animals. This result was considered in favor of the hypothesis of an intimate relation between the condition of the pulmonary arch and gill reduction (see Figs. 3 and 4).

THE MORPHOLOGY OF THE PULMONARY ARCH IN PERENNIBRANCHIATES

Since an attempt has recently been made to create the impression that all six aortic arches appear in *Necturus* (Shumway, Olson, and Kugler, 1930), it appears necessary to discuss briefly the work on the morphology of the sixth aortic arch before reporting on a series of new experiments.

The absence of the sixth aortic arch has been reported in several older papers. Huxley in 1874, describing the condition in *Necturus* (at that time called *Menobranchus*), mentions that no trace "of a fourth epibranchial" is found in *Necturus;* he also noticed that only three aortic arches were present. Moreover, he noticed the similarity of this condition in *Necturus* and *Proteus*, as described by Van der Hoeven, 1867. Boas (1882) gives a complete picture of the aortic arches in *Necturus*. He observed the absence of both the pulmonary arch and the spiral valve in *Necturus* and described fully the same condition in *Proteus*, another perennibranchiate amphibian. He also deserves credit for being the first one to suspect that the sixth arch and spiral valve represent an indispensable mechanism in the metamorphosis of amphibians. More recently Wilder (1895) described and illustrated clearly the reduction of the sixth visceral arch in *Necturus*.

A third perennibranchiate amphibian has since been described in *Typhlomolge rathbuni*. It is of considerable interest that in this animal the most caudal or pulmonary arch is also absent (Uhlenhuth, 1923). Evidently the true perennibranchiate amphibians, while they may be a heterogeneous group in many respects, all show a reduction of the ventral portion of the pulmonary arch.

Since 1923 no reference has been made to the anomalous condition of the sixth aortic arch in perennibranchiate amphibians, until it was described in *Necturus*, in a short preliminary report, without the knowledge that the observation had been made by earlier investigators (Figge, 1928). This observation, together with the result of ligation experiments on the tiger salamander, was reported at the meeting of anatomists, March 27–30, 1929.

Shortly after this, Noble (April, 1929) reinvestigated and confirmed the observed reduction in the number of visceral and aortic arches in *Necturus*. He found that, even in stages shortly after

hatching, the branchial arches have the same reduced number and form as in the adult.

Shumway, Olson, and Kugler (1930) claim that the ventral portion of the sixth aortic arch is represented by a vestigial ventral twig found in embryos between 20–40 mm. in body length, and a dorsal twig arising as in *Ambystoma* and from which the pulmonary artery originates. The "dorsal twig" or "dorsal portion of the sixth aortic arch" has been more correctly referred to in earlier papers as the ductus arteriosus (Figge, 1930). The fact that a vestige of the ventral portion of the sixth aortic arch may sometimes be found in the early stages would be quite interesting from the standpoint of the biogenetic law. In this light, either its appearance and then disappearance, or occasional occurrence, may be considered as further evidence for the theory that *Necturus* has undergone retrograde development. The report of Shumway and his co-workers is at hand only in abstract form, and the number of cases in which the vestigial ventral twig was observed is, therefore, not known.

When it was first noticed (January, 1928) that the ventral portion of the sixth aortic arch was absent, it was suspected that a vestige of this might occasionally be found. Twenty adult *Necturi* were, therefore, injected with a gelatine solution of various colors in an attempt to find such a vestige. According to the notes made at that time, no trace of it was found; but in two specimens a very small vessel was found in the normal position of the sixth aortic arch which seemed to take its origin from the bulbus arteriosus. These results were not published at that time because the connection to the bulbus arteriosus could not be satisfactorily demonstrated. Since then it has been possible to examine living *Necturi* in the egg, the hatching, the 40 mm., and the larger sizes. When the living animals were carefully dissected with the aid of a carbon arc light and a dissecting microscope, it became evident that the vessel which appears to be a ventral twig or vestige of the sixth aortic arch is, in reality, a small vein. This vessel has very thin walls, and the blood in it flows more slowly than in an arterial vessel and does not pulsate. The one on the right side usually pierces the pericardium and is continuous with a vessel passing along the dorsal side of the bulbus arteriosus. In no case did I find a vessel homologous to the ventral portion of the sixth aortic arch.

But, even if we assume that the "ventral twig" described by Shumway was the vestige of the sixth arch, it was too small to be of any consequence. It should be pointed out that it does not connect with the dorsal portion (ductus arteriosus or pulmonary artery) and, strictly speaking, does not form an aortic arch. For the same reason it could not develop into a normal pulmonary artery carrying blood directly from the heart to the lungs; so that, even if the observation of Shumway and his co-workers is correct, it has no bearing on the theory in regard to the retention of gills in perennibranchiates. It is doubtful, however, that the twig described by Shumway, Olson, and Kugler is a vestige of the ventral portion of the sixth aortic arch.

Very recently Shumway seems to have changed his views. In discussing the chondocranium of a 23 mm. *Necturus*, he mentions especially the fact that he was unable to distinguish any trace of a fourth branchial (sixth visceral) arch. Shumway and Webb (1932), while they do not mention in this paper the vestige of the ventral portion of the sixth aortic arch, at least confirm the anomalous condition of the visceral arches, which is contrary to the impression Shumway, Olson, and Kugler gave in the earlier paper.

THE PURPOSE OF THE FOLLOWING EXPERIMENTS

So far no one has actually repeated the ligation experiments under exactly the same conditions under which they were originally performed by the author. Attempts have, nevertheless, been made by Helff (1931), Garber (1930), and Ichikawa (1931) to devise similar experiments. The results of their work are interpreted by these investigators as evidence disproving the aforementioned theory in regard to retention of gills on *Necturus*. In spite of the claims made for these experiments, however, they are not comparable to the ligation experiments of the writer, as will be shown later.

The experiments on *Ambystoma tigrinum* (Colorado axolotl) which were performed in Colorado were only qualitative; and it was suggested that the work be repeated quantitatively. The experiment was repeated three times, with minor modifications in each case. The present paper is a report of the results obtained in these three experiments. In the first of them it will be shown that the effect of ligation of the pulmonary arch on metamorphosis is much different in this environment (Baltimore) from what it was in the environment main-

tained in earlier experiments (Colorado Springs). The second experiment is an attempt to determine which of the factors in the two environments were contributing most to the differences in results. The third experiment is an attempt to demonstrate the quantitative nature of the effect of ligation of the pulmonary arch on the metamorphosis of *Ambystoma tigrinum* larvae.

MATERIAL

The Colorado axolotls used in these experiments were obtained through the courtesy of Dr. R. J. Gilmore. They were all collected near Colorado Springs and shipped in cans that were kept iced during the journey. But, in spite of the fact that they were kept cool, they always showed the usual metamorphic stimulus due to environmental changes caused by shipping.

The thyroid powders used in Experiments I and III were obtained from Armour and Company through the courtesy of Dr. Frederick Fenger. The anterior lobe powder used in Experiment III was prepared in the laboratory by Uhlenhuth's method, which will be described later.

METHODS

The ligation of the sixth aortic arch is a relatively simple matter. It involves no cutting or bleeding. The animal is anesthetized in chloretone. The sixth arch is exposed by merely raising the gular fold. Cephalad to the sixth visceral arch is a gill slit; caudally there is no slit, because it is attached to the skin and a muscle layer. It is, therefore, necessary to pierce this skin and muscle layer (which is very thin) just caudal to the visceral arch in order to pass the ligature. Cephalad, the ligature is passed through the gill slit and tied. It thus passes around both the aortic and visceral arch. The positions of the ligatures are indicated in Figure 4. The operated animals are referred to in this paper as "ligated animals," and only the ventral portion of the sixth aortic (pulmonary) arch is ligated.

In tying the ligature, it has been found best to tie it just tight enough so that it does not cut through the visceral cartilage. Dental floss was found to be a very efficient ligature for the operation because it may be flattened into a ribbon. It is placed in 70 per cent alcohol to soak before using. This ribbon-like ligature has the advantages of being strong, of not being absorbed, and may be tied

tightly without cutting the visceral arch. Other types of ligatures frequently cut the visceral arch, and the animals bleed considerably and frequently die. (If kept in very cold running water they may survive the bleeding; but even so, this is not a desirable factor to introduce in this experiment.)

It is essential that two ligatures be made on each arch, four per animal. In Experiment I, ten ligatures were made on each animal. Two dental floss ligatures were put on each arch about 7–8 mm. apart. Then two rubber-band ligatures were put over these, insuring a constant tension and no bleeding. Then a ligature of 30-day chromic catgut was put on between the two. This may be so tight as to cut the visceral arch. In Experiments II and III the rubber bands were omitted, only three ligatures being placed on each arch—two dental-floss ligatures at the extreme dorsal and ventral ends of the visceral arch and one of chromic catgut between these. The catgut ligature was pulled tightly enough to cut the visceral arch and, necessarily, put on last.

The actual ligation takes only a few minutes per animal. The visceral arches and blood vessels eventually fall out and are usually found in the container along with the ligatures. This is rarely accompanied by bleeding if the animals are kept cool (below 15° C. or in running water). It was noted that animals that had started to metamorphose before they were ligated have a tendency to bleed. The same tendency was noticed in animals kept at temperatures above 18° C. Some animals may be ligated and not bleed for months; the ligature and visceral arches may even fall out without bleeding. Yet the same animals may almost bleed to death from the ventral stub of the sixth arch when anterior lobe or thyroid extracts are injected or when subjected to negative pressure.

The animals in Experiment I were fed twice during the experiment. The animals in Experiments II and III were not fed during the entire experimental period (about one year). The examination or injection of the animals involved anesthetizing them in 0.1 per cent chloretone solutions until they did not move when handled. After the examination or injection they were revived in running water.

The anterior-lobe powder used in Experiment III was prepared by the method used by Uhlenhuth. The anterior and posterior lobes

were carefully separated, as suggested by Spaul (1925 *c*, 1930), within 1 hour after the cattle were killed. The anterior lobes were then cut into small pieces, air-dried, and ground to a powder. The powder is stored in a desiccator in the ice chest until needed.

The extraction of the "thyreo-activator" (Uhlenhuth) from the anterior-lobe powder was done in the same hour that the injections were made. The extractions were made with 0.5 per cent glacial acetic acid in 30 per cent frog Ringer. This was placed in a small flask with a weighed amount of anterior-lobe powder and heated to the boiling-point and then immediately filtered. The extract was then neutralized with N/5 NaOH against litmus paper. The extraction process was so regulated that after neutralization, 0.5 cc. of extract was obtained for each 100 mg. of anterior-lobe powder used.

The thyroid powder was extracted with 30 per cent frog Ringer. This was placed in a flask with a weighed amount of powder brought to the boiling-point and filtered.

The animals in Experiments I and III were injected thrice weekly on alternate days. The dosage for thyroid injections varied and will be given with the individual experiment. The dosage of anterior-lobe extract was 0.5 cc. of extract (100 mg. of anterior lobe) per animal per injection.

An environment of negative pressure (low oxygen tension) was maintained for the animals by placing them in individual one-half gallon bottles with No. 12 one-hole rubber stoppers. Through the hole in the stopper there was placed a short piece of glass tubing; and on the end of this, a short rubber tube with a screw clamp. The bottles were filled about one-half full of water, and the air aspirated by means of a water pump until the pressure, as recorded by means of a mercury manometer, registered −16 cm. The bottles were then closed by means of the screw clamp and placed in the refrigerator.

EXPERIMENT I

THE EFFECT OF LIGATION OF THE PULMONARY ARCH ON METAMORPHOSIS OF AMBYSTOMA TIGRINUM AT SEA LEVEL

SPECIAL EXPERIMENTAL CONDITIONS

Twelve Colorado axolotls were received from Colorado on April 2, 1931. The animals were fairly large, measuring 185–210 mm. (average 200 mm.). Six of these were ligated on April 4, 1931. They were

kept in four tanks of running water, varying in temperature between 17° C. and 22° C. (average 18° C.). They were given numbers as follows:

Group I	Aa	(animals 1, 2, 3)	Non-ligated	Non-injected
Group II	Ab	(animals 1, 2, 3)	Non-ligated	Thyroid-injected
Group III	Ba	(animals 1, 2, 3)	Ligated	Non-injected
Group IV	Bb	(animals 1, 2, 3)	Ligated	Thyroid-injected

It will be noticed that the animals are of two types: (A) control or non-ligated; (B) ligated. There are likewise two types of metamorphosing stimuli. The thyroid injection is by far the most potent. The other is the change in the environment. This involves shipping from a high altitude in Colorado to near sea-level in Maryland. Whatever the factors involved, the latter is a fairly potent metamorphosing stimulus and had the advantage of being exactly the same for all animals. Any variations in regard to the onset or rate of metamorphosis when this stimulus alone is applied must, therefore, be due to individual variation within the animals. While there is some variation in the metamorphosis of normal animals, none of these have failed to metamorphose with this stimulus.

The animals in Groups I and III received only the latter stimulus, while those in Groups II and IV received, in addition, the injection of thyroid extracts.

The thyroid injections were started May 2, 4 weeks after the operations. This was considered sufficient time for the animals to recover from the operation. (Animals bleed from ventral stub of sixth arch if injected too soon.) They were examined at this time with a dissecting microscope, and it was recorded that the aortic and visceral arches between the ligatures had dropped out. Even the ligatures were not present, and no evidence of a regenerated blood vessel could be found. The thyroid injections were made on alternate days. The injected animals (Groups II and IV) received seven injections. Each animal received the extract of 30 mg. thyroid powder in the first four injections; in the last three injections the dosage was increased from 30 mg. to 40 mg. per injection per animal. The last injection was given on May 16, 1931.

RESULTS OF EXPERIMENT I

On the fourteenth day after the injections were started, the animals in Group II Ab, the non-ligated, thyroid-injected animals, were

PLATE III

Photographs of a representative animal from each group in Experiment I. These show the retarding effect of the ligation in the metamorphosis of animals kept at 18°–22° C. and high oxygen tension, 76 cm. pressure.

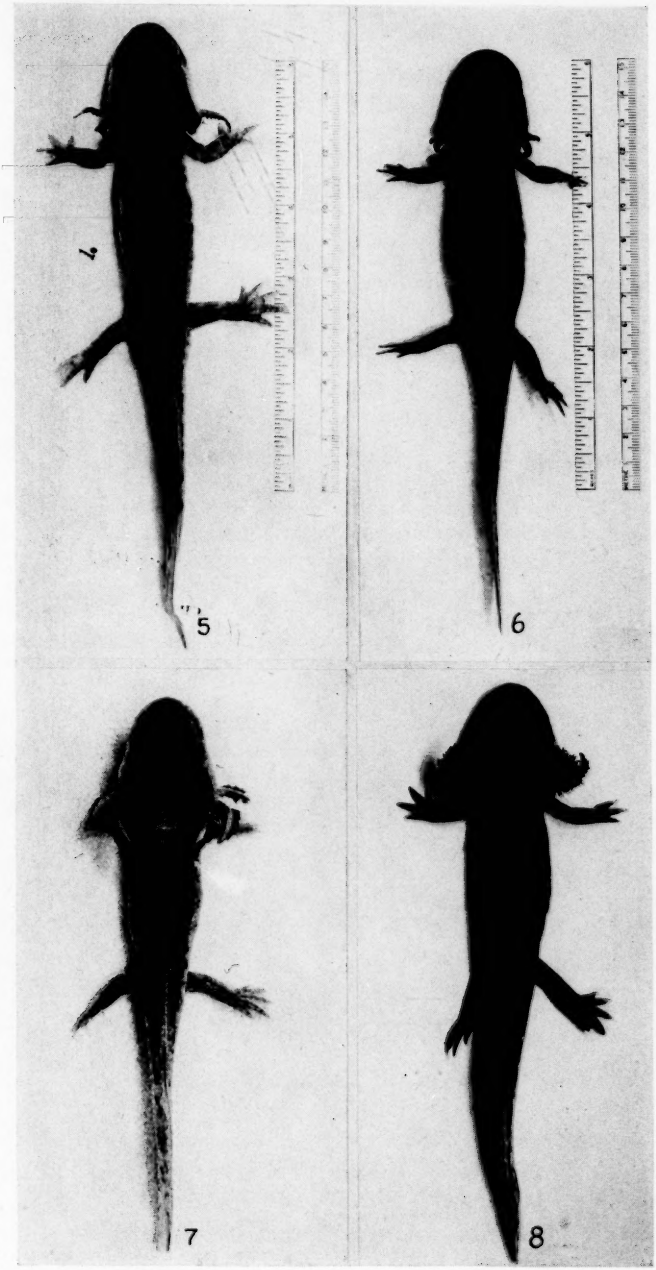
FIG. 5.—A non-ligated, non-injected animal, Aa_1 (Group I). Note partial reduction of gills and gill filaments.

FIG. 6.—A non-ligated, thyroid-injected animal, Ab_2 (Group II). Note a reduction of gills to stubs.

FIG. 7.—A ligated, non-injected animal, Ba_1 (Group III). Note no reduction of gills. (Spots were present at time of ligation.)

FIG. 8.—A ligated, thyroid-injected animal, Bb_2 (Group IV). Note the large gills and gill filaments and the manner in which the gills curve forward.

PLATE III



far advanced in metamorphosis and were completing the process rapidly. The injections were discontinued on this day.

The non-ligated, non-injected animals of Group I were rapidly metamorphosing but had not advanced as far as the injected group. One animal in this group deviated considerably in regard to the rate of gill reduction. These were not being reduced by the fourteenth day in spite of the fact that some of the adult coloration had appeared and the fins were reduced. Group IV, the ligated, thyroid-injected animals, had previously shed skin, but the gill filaments were of great size. The gills were turned forward and waving with each heart beat (see Fig. 8). In the ligated, non-injected animals of Group III Ba, no changes had been noted. The gills were in excellent condition.

On the sixteenth day after injections were started (2 days after injections had been discontinued), the animals were all photographed, the conditions being practically the same as noted for the fourteenth day. Figures 5, 6, 7, and 8 are photographs of a representative animal of each group and clearly show the differences in regard to the reduction of gills. The groups arranged in order of gill reduction are:

Most reduced	Group II	Ab	Non-ligated	Thyroid-injected	Fig. 6
Reduced	Group I	Aa	Non-ligated	Non-injected	Fig. 5
Not reduced—curled forward	Group IV	Bb	Ligated	Thyroid-injected	Fig. 8
Not reduced	Group III	Ba	Ligated	Non-injected	Fig. 7

At the time the photographs were made (16 days after injections were started), and even 2 or 3 days later, it seemed that the experiment would be a duplication of the ones performed in Colorado. The controls or non-ligated animals, both injected and non-injected, were rapidly metamorphosing. The ligated non-injected animals had not changed. The ligated thyroid-injected animals had gills turned forward in a manner which is characteristic of ligated animals that have received a strong metamorphic stimulus. The gill filaments, though still quite large, were beginning to twist and shrivel at the tips. This group of animals seemed to be at the same critical stage in which the ligated animals in Colorado had died. It was thought that if the gill filaments were further reduced, these animals would likewise not survive.

On the twentieth day after injections were started, the animals of the ligated, thyroid-injected group shed skin, and not only continued metamorphosis but did so very rapidly (although injections had been discontinued for 4 days). Three days later the gills were mere stubs, and on the twenty-sixth day they were mere nodules. This group soon resembled the non-ligated controls. Moreover, the ligated, non-injected animals also started to metamorphose. The first signs of metamorphosis (skin-shedding) were noted on the twentieth day. The gills began to show signs of reduction on the twenty-third day, and by the thirtieth day the gills were mere stubs.

The aortic arches of the ligated animals were carefully dissected to see if the pulmonary arch had regenerated. No regeneration was ever found.

DISCUSSION OF EXPERIMENT I

Briefly, all the animals in this experiment metamorphosed regardless of the ligation. The effect of the ligation was evident only in the retardation of the onset of the metamorphosis in general and gill reduction in particular. The ligated animals did not begin metamorphosis until the corresponding controls were almost metamorphosed.

These results differ from those obtained in Colorado and make it necessary to modify an earlier statement, namely, "that the ventral portion of the sixth aortic arch is necessary for metamorphosis to take place in *Ambystoma tigrinum*." It is evident, however, that obliteration of the ventral portion of the pulmonary arch by ligation produces a specimen that is less sensitive to metamorphosing stimuli or substances than the normal or non-ligated animal.

EXPERIMENT II

THE ANALYSIS OF THE ENVIRONMENTAL FACTORS IN RELATION TO THE METAMORPHOSIS OF LIGATED ANIMALS

While the results obtained in the foregoing experiment differ from those obtained in Colorado, the experimental conditions were also quite different. The temperature of the running tap water was roughly 7°–10° higher in Baltimore than in Colorado Springs. The tap water in Colorado Springs is derived from the melting snow on Pikes Peak watershed and comes to the city almost as pure as distilled water. The oxygen tension is considerably higher in Baltimore than in Colorado Springs (Baltimore, barometric pressure 76

cm.; Colorado Springs, 60 cm.). Other differences may exist that are not suspected. In this experiment an attempt was made to determine whether any of the preceding factors were related in any way to the effect of the ligation.

SPECIAL EXPERIMENTAL CONDITIONS

Twenty-four animals were received from Colorado on August 10, 1931. They were kept iced during the journey. The animals were quite large (200–240 mm.), and all were in good condition on arrival; but notwithstanding the attempt to avoid the metamorphosing stimulus by keeping them cool, they showed pre-metamorphic symptoms —that is, the gill filaments were very short at the tips and there was a slight reduction of the ventral fin. Sixteen animals were put into stone crocks containing tap water and placed in a refrigerator at 6°–10° C. Eight animals were kept in stone crocks at higher temperatures (22°–26° C.). Metamorphosis of the animals, though it continued, was somewhat retarded by the lower temperatures. During the entire experiment the water was precooled for the animals kept at low temperature.

Twelve animals were ligated on August 15, 1931. These were placed in individual crocks and subjected to various environmental conditions. The variables employed in maintaining the artificial environments were: the type of water, the temperature, and the oxygen tension or air pressure. The animals were numbered A 1–12, non-ligated, and B 1–12, ligated. The accompanying chart indicates the type of environment maintained for each animal (see Table I). For each ligated animal there is a control or non-ligated animal of the same number kept under exactly the same conditions.

The animals were not injected, since they had already started the process of metamorphosis; and from the results in the preceding experiment it was questioned whether the ligation would stop this. The results will not be given in detail for each animal, but arranged in tabular form (see Table II) so that they may be discussed in groups.

RESULTS OF EXPERIMENT II

Group I.—Animals kept in distilled water at high temperature (22°–26° C.) and at 76 cm. pressure.

It was evident that the temperature was too high for this group.

Owing to the fact that this was a very potent metamorphosing stimulus, the non-ligated animals (A 5, 6, 11), began metamorphosing

TABLE I

SUMMARY OF RESULTS IN EXPERIMENT II

Animals grouped according to environment or corresponding conditions.

KEY: Metamorphosed c. Completed metamorphosis
Metamorphosed p. Partial metamorphosis
A animals Non-ligated
B animals Ligated

Group	Condition	Animals	Results	Days	Remarks
I.....	Distilled water	A5	Metamorphosed p.	10	Died
	22°–26° C.	A6			Died first day
	76 cm. pressure	A11	Metamorphosed c.	15	
		B5	No change. Bled	9	Died larval
		B6	No change. Bled	12	Died larval
		B11	No change. Bled	14	Died larval
II.....	Running tap water	A12	Metamorphosed c.	15	
	18°–22° C.				
	76 cm. pressure	B12	Metamorphosed c.	294	Examined
III....	Distilled water	A1	Metamorphosed c.	72	
	6°–10° C.	A2	Metamorphosed c.	70	
	76 cm. pressure	A7	Metamorphosed c.	73	
		A8	Metamorphosed c.	73	
		B1	No change	403	Still alive and larval
		B2	No change	403	Still alive and larval
		B7	No change	403	Still alive and larval
		B8	No change	320	Died larval
IV....	Tap water	A4	Metamorphosed c.	77	
	6°–10° C.	A10	Metamorphosed c.	27	
	76 cm. pressure				
		B4	No change	403	Still alive
		B10	Skin Shed	393	Died larval?
			Slight gill reduction		
			These regenerated		
V.....	Distilled water....	A3	Metamorphosed c.	74	
	6°–10° C.	A9	Metamorphosed c.	74	
	60 cm. pressure				
		B3	No change	35	Bled. Died
		B9	No change	43	Large gills

very rapidly. One of these died early; another that was almost adult died on the tenth day (drowned); and the other was considered

metamosphosed on the fifteenth day. The ligated, or "B," animals did not continue metamorphosis. The gills, on the contrary, became quite large and distended, waving slightly with each increase in blood pressure. The animals appeared to be uncomfortable, and all bled considerably from the ventral stub of the pulmonary artery. They all died in larval stage with very large gills. It was noted that the non-ligated animals had shed skin several times and had reduced the gills to mere stubs before any of the ligated animals died.

Group II.—Animals kept in running tap water at 22° C. and at 76 cm. pressure.

These conditions correspond to those in the preceding experiment. There were only two animals kept under these conditions. The non-ligated animal, A 12, metamorphosed in 15 days; the ligated animal, B 12, remained larval for a long time and eventually metamorphosed on the two hundred and ninety-fourth day. This was the only ligated animal in this experiment that metamorphosed.

Group III.—Animals kept in distilled water at 6°–10° C. and 76 cm. pressure.

The non-ligated, or "A," animals (1, 2, 7, 8) in this group all metamorphosed. The process was considerably retarded by the low temperature, as evidenced by the fact that this took about 72 days in each case, as compared with 15 days in the groups at higher temperatures. In the ligated animals kept under the same conditions, metamorphosis was completely suppressed. One of these even regenerated a portion of a gill that had been chewed off. Animal B 8, which died on the three hundred and twentieth day, had very large gills and was larval in every respect. The other ligated animals in this group (B 1, 2, 7) are still alive and have not even shown a tendency to metamorphose at the time of this writing, 403 days after the ligation.

Group IV.—Animals kept in tap water at 6°–10° C. under atmospheric pressure (76 cm.).

The "A," or non-ligated, animals (A 4, 10) both metamorphosed, one at about the same rate as those in Group III (74 days) and the other more rapidly (27 days). The "B" animals (B 4, 10) did not metamorphose. Animal B 4 showed no change whatever and is still living (403 days after ligation). The other animal, B 10, remained

larval in most respects for 393 days after ligation, when he died. The record of this animal is of interest because of the strange reaction. The gills were in excellent condition at the time of ligation, August 15, 1931. On October 19, 75 days later, the animal began shedding skin. Ten days later it was noted that the gills were shortening and the gill filaments were shriveling, while skin-shedding in large pieces continued. The skin-shedding and gill reduction continued for about 2 months, during which time it was thought the animal would metamorphose. When it was noticed that the skin-shedding had stopped, the gills were measured and found to be about half their original length. Many of the gill filaments were completely shriveled, and the ones present were sparse and scraggily looking. The dorsal body fin had been completely reduced and the tail fins partially. The skin had become peculiarly rough, and fine wrinkles appeared when the animal moved about or assumed a curved position. During the next 6 months no skin was shed and the gills seemed to grow longer. The gill filaments also seemed to grow larger and even increase in number, but they were not actually counted. The gills were measured again at the end of this period and were found to have increased in length 4–6 mm. per gill.

Group V. Animals kept in distilled water at 6°–10° C. at negative pressure (76 − 16 or 60 cm.).

This set of experimental conditions more closely resembles the Colorado environment. It will be seen that the "A" animals (A 3, 9) metamorphosed at about the same rate as the other non-ligated animals in low temperatures (74 days). The ligated animals (B 3, 9) died on the thirty-fifth and forty-third days, respectively. Both had bled from the ventral stump of the sixth arch. They had not shown any metamorphic changes; on the contrary, the gills had become quite large and turned forward. It was noted that when the air in the negative pressure jars was not changed each day, the gill filaments were greatly distended with blood and seemed almost unbelievably large. The gill filaments on B 9 were actually found bleeding. At that time one of the largest gill filaments measured 8 mm. in length.

To summarize: All non-ligated "A" animals metamorphosed regardless of the environment except one, which died on the first day.

The rate of metamorphosis, however, is controlled by the temperature. None of the ligated "B" animals kept under corresponding conditions metamorphosed except B 12, the animal kept under conditions simulating those in the preceding experiment. Yet the retarding effect of the ligation was in this case demonstrated quite clearly, as this ligated animal needed two hundred and ninety-four days to metamorphose, as compared to 15 days for the controls.

DISCUSSION OF EXPERIMENT II

The reaction of B 10 (partial metamorphosis) and B 12 (complete metamorphosis) would indicate that the tap water contained something that stimulates metamorphosis. But B 4 was also in tap water in the same group as B 10 and did not metamorphose. The evidence that there is any difference between the tap water and distilled water in regard to its effect on metamorphosis of ligated animals is, therefore, insufficient.

The temperature is the factor that has the most decided effect on the metamorphosis of ligated animals. The temperature controls the rate of, and tendency to, metamorphose in a normal animal. In general it may be said that the higher temperatures accelerate metamorphosis while the lower temperatures retard this. It is thus possible to resort to temperature regulation as a means of controlling the potency of a metamorphic stimulus. The temperature maintained for Group I, 22°–25° C., was evidently very potent, since normal animals metamorphosed quite rapidly. For the ligated animals, this was too high and they bled considerably. The gills were greatly distended and death resulted. Had these animals been in well-oxygenated, running water at this temperature, they may have survived and eventually metamorphosed, as did B 12 at a slightly lower temperature. At temperatures of 6°–10° C., the effect of ligation on metamorphosis is most pronounced (Groups III, IV, V). This temperature is low enough to retard metamorphosis in the non-ligated animals and completely inhibit it in the ligated animals.

The negative pressure or low oxygen tension had a decided effect on the gills, causing their enlargement. A retarding or inhibiting effect on the metamorphosis of ligated animals could not be demonstrated, as the ligated animals did not survive the lowered pressure. (The controls under negative pressure metamorphosed.)

EXPERIMENT III

THE QUANTITATIVE NATURE OF THE EFFECT OF LIGATION

The preceding experiments show that the effect of the ligation varies considerably with the environment or external factors. The effect is quantitative, at least as far as temperature is concerned. At 22° C., ligation merely retards metamorphosis. At 6°–10° C., the effect of ligation is most pronounced; metamorphosis is completely inhibited. This may be only indirectly due to the low temperature, which may merely inhibit the production or release of the metamorphosis-stimulating hormones.

In the following experiment an attempt was made, therefore, to investigate whether the internal secretions show a quantitative relation to the effects of ligation. The animals were kept at low temperature only, or at low temperature combined with negative pressure. Thyroid hormone was injected, and the relative effects of corresponding quantities in ligated and non-ligated larvae were recorded. It was desirable to know in this connection whether the animals' own thyroid would be able to produce and release a sufficient amount of hormone to metamorphose the ligated animals. The thyroids were activated by means of injections of thyreo-activator extracted from the anterior lobe (Uhlenhuth).

SPECIAL EXPERIMENTAL CONDITIONS

The animals for this experiment arrived from Colorado on October 17, 1931. They were put in tap water and immediately assorted, and then put in distilled water in stone crocks and placed in the refrigerator at 6°–10° C. They were all in good condition, but they had started to metamorphose. They continued this process for 2 or 3 days in spite of the lower temperatures.

On October 20, twenty of the largest animals were chosen and placed in individual glass jars. They ranged in size from 165 to 200 mm. (average length 186 mm.). Eight of these were ligated on the same day, and seven on the next. All of the animals were kept at the same temperature throughout the experiment. This was found to fluctuate between 6° and 12° C. It should be stated, however, that it was necessary to raise the temperature for short periods during injections. During this time they were anesthetized lightly in chlore-

tone, injected, and placed in running tap water (temperature 16°–20° C.) until they recovered. They were then returned to the refrigerator.

Two kinds of gland extracts were injected—thyroid and anterior lobe. The dosage for the thyroid was not the same for all injections. Small doses were used at first, and these increased in an attempt to regulate the stimulus so that the ligated animals at negative pressure might metamorphose at a rate favorable for their survival.

The activity of the anterior-lobe extracts is demonstrated in this experiment by the fact that the controls metamorphosed and that it produced abundant skin-shedding in the ligated animals.

The injections were started (January 1, 1932) 71 days after ligation. Eight ligated and four non-ligated animals were injected. The results and the conditions are summarized in Table II. The animals designated "A" are non-ligated, and those designated "B" are ligated. Seven of the ligated animals were not injected; four of these were kept at low pressure.

RESULTS OF EXPERIMENT III

In Group I (see Table II) the number of milligrams of thyroid powder necessary to produce any given metamorphic reaction in ligated animals is invariably greater than the number of milligrams necessary to produce the same change in a non-ligated animal. The ratios of the amounts necessary to produce the specified changes are: ligated/non-ligated; to produce skin-shedding, 300/120; to reduce gill filaments, 480/220; to reduce gills to mere stubs, 550/300. While these ratios show that it is necessary to inject a greater amount of thyroid extract in a ligated than in a non-ligated animal to produce the change, they are not a true indication of the effect of the ligation because the time factor is not considered. If this were taken into account, the differences would be even greater, for it required not only more extract to produce a specified change but also more time.

The injections were discontinued after all the non-ligated animals had metamorphosed. At that time the ligated animals had reduced the gills to stubs and shed their skins. But even without further thyroid injections they finally underwent complete metamorphosis just like the controls.

TABLE II

SUMMARY OF RESULTS IN EXPERIMENT III

Conditions and number of milligrams of dried gland substance necessary to produce various metamorphic changes in "A," non-ligated, and "B," ligated, animals.

KEY: A.L. Anterior lobe
Cm.P. Centimeters pressure
G.N. Gill nodules
G.S. Gill stubs
Meta. Metamorphosed
Not inj. Not injected
R.G.F. Reduction of gill filaments
S.S. Skin-shedding
Thy. Thyroid

Animals	Conditions		Milligrams of Gland Powder Necessary To Produce				Total Injected in Milligrams	Results
	CmP	Type of Injection	S.S.	R.G.F.	G.S.	G.N.		
Group I:								
A2	76	Thy.	120	220	300	380	480	Meta.
Bb1	76	Thy.	300	480	530	.....	580	Meta.
Bb2	76	Thy.	300	480	580	.....	580	Meta.
Group II:								
AA1	60	Thy.	60	220	300	380	480	Meta.
Bb1	60	Thy.	300	580	Died at R.G.F.		580	Died
Bb2	60	Thy.	Died after fourth injection. Bled				120	Died
Group III:								
A1	76	A.L.	600	700	1,200	1,800	2,000	Meta.
Bb3	76	A.L.	800	Gills not reduced			2,700	Larval
Bb4	76	A.L.	800	Gills not reduced			2,700	Larval
Group IV:								
AA2	60	A.L.	600	800	1,500	2,000	2,000	Meta.
BBb3	60	A.L.	800	Gills not reduced			2,700	Larval
BBb4	60	A.L.	800	Gills not reduced			2,700	Larval
Group V:								
B1	76	Not inj.	No change					
B2	76	Not inj.	No change					
B3	76	Not inj.	No change					
Group VI								
BB1	60	Not inj.	No change					
BB2	60	Not inj.	No change					
BB3	60	Not inj.	No change					
BB4	60	Not inj.	No change					

The animals in Group II were treated in exactly the same manner as those in Group I, except that they were kept at negative pressure (60 cm.). The non-ligated animal AA1 metamorphosed at about the same rate as the corresponding animal in Group I (skin-shedding occurred slightly earlier). One of the ligated animals bled considerably and died after the fourth injection. The other ligated animal (BBb2) shed skin at about the same time and with the same amount of thyroid as the ligated animals of the preceding group. The gills were, however, more resistant; it required 580 mg. of thyroid powder to start the reduction of gill filaments. Unfortunately this animal died just after the gill filaments began to shorten. The ratio of the number of milligrams of thyroid to produce reduction of gill filaments is in this case: ligated/non-ligated: 580/220, slightly higher than for the preceding group.

The results in Groups III and IV may be discussed together. These groups correspond to Groups I and II, but thyreo-activator, extracted from bovine prehypophysis was injected in place of thyroid. It may be seen that the "A," or non-ligated, animals metamorphosed, while the "B," or ligated animals, shed their skins only but did not reduce the gills. The ligated larvae, however, received three times the amount of anterior-lobe powder necessary to reduce the gills of the non-ligated larvae. Moreover, the amount of anterior-lobe powder necessary to produce skin-shedding in the ligated was 800 and in the non-ligated, 600.

Group V and VI, consisting of ligated animals, were kept under the same conditions as the other animals but not injected. No changes were noted.

DISCUSSION OF EXPERIMENT III

It is evident that the amount of thyroid extract necessary to produce any given metamorphic change in a ligated animal is far greater than the amount necessary to produce the same change in a non-ligated animal. If the temperature is sufficiently lowered, the animal's own thyroid cannot manufacture enough thyroid hormone to produce metamorphosis after ligation. The latter circumstance accounts for the fact that ligation of the sixth gill arch prevents metamorphosis completely if the animals are kept at low temperature.

Furthermore, it is of interest that at low temperature combined with low air pressure metamorphosis cannot be produced at all, even if thyroid extracts are administered. The animals die before metamorphic changes appear.

DISCUSSION

The experiments reported above demonstrate that temperature is one of the most important factors in determining the effect of the ligation on metamorphosis. The relationship of temperature to the effect of the ligation is, however, an indirect one. This is due, no doubt, to the fact that the temperature is directly related to the tendency of the animal to metamorphose, or the potency of a metamorphic stimulus in a normal animal. By keeping ligated animals at a low temperature (6°–12° C.), they are subjected to a very weak metamorphosing stimulus which is just above the threshold necessary to metamorphose a non-ligated animal but just below the threshold necessary to metamorphose a ligated animal. The effect of the ligation on metamorphosis is, therefore, most evident in an environment where the temperature is low. If we increase the temperature (Experiment I, and Experiment II, B 12), the stimulus exceeds the threshold necessary to metamorphose ligated animals and they metamorphose, provided they are kept in an environment of high oxygen tension.

The internal secretions bear a similar relation to the effect of the ligation. Even ligated animals may be metamorphosed in an environment of low temperature, if enough thyroid extract is injected and the oxygen tension in the environment is high enough. The injection of thyreo-activator extracted from the anterior lobe acts as a metamorphic stimulus, potent enough to produce metamorphosis in a normal animal at low temperature but not in a ligated animal. In the ligated animals it was impossible to stimulate the animal's own thyroid to produce or release enough thyroid hormone to exceed the threshold necessary to produce metamorphosis at low temperatures.

The results of the previous experiments performed at the altitude of 6,048 feet and the present three carried out at sea-level with variable oxygen tension indicate that the effect of the ligation of the sixth aortic arch on metamorphosis depends considerably upon the

oxygen tension in the environment of the animals. This factor seems to be more directly related to the effect of the ligation on gill reduction. While it was not possible in this experiment to determine the relation of oxygen tension to the effect of the ligation quantitatively, the qualitative results clearly show that this is the factor in any environment that determines whether or not a ligated animal may be successfully metamorphosed. The list of ligated animals that completed metamorphosis shows that they were all kept in an environment of high oxygen tension:

a)	6 ligated animals in Exper. I	Running water	76 cm. pressure
b)	1 ligated animal in Exper. II	Running water	76 cm. pressure
c)	2 ligated animals in Exper. III	Low temperature	76 cm. pressure

None of the ligated animals at negative pressure metamorphosed. In the three experiments an attempt was made to metamorphose a total of six ligated animals in an environment low in oxygen tension. In each case the attempt was a failure. They do not react to an average metamorphic stimulus; and when the strength of the stimulus is increased, the animals do not survive. The attempts to metamorphose twelve ligated animals at an elevation of 6,048 feet in previous experiments gave similar results. This is additional evidence in favor of the hypothesis that gill reduction is in some way related to the oxygen intake at points other than the gills.

Some experimental evidence has recently been advanced to disprove the hypothesis that the retention of gills in perennibranchiates is due to the failure of the pulmonary arch to develop. In this connection the work of Ichikawa (1931) is of interest. He used the larvae of *Hynobius*, a small form of about 45–50 mm. in length. The sixth aortic and visceral arch were removed, resulting in practically the same conditions as that produced by the ligation described in this and previous papers (Figge, 1930). The *Hynobius* larvae, however, all metamorphosed.

It is, unfortunately, very difficult to analyze critically the work of Ichikawa, as he omits the records of some of the most pertinent conditions. He does not mention the temperature at which the animals were kept, and from the experiments reported here it is evident that the temperature is one of the most important factors in determining the effect of the ligation. Moreover, it appears that Ichikawa does

not fully appreciate the problems involved in the effect of ligation upon metamorphosis; otherwise, it would be difficult to understand why he should bubble oxygen through the water in which the ligated animals were kept. This procedure alone may be responsible for the negative results he reports.

Finally, the effect of the ligation may be closely related to the size of the animal. Owing to the small size of the *Hynobius* larvae, the surface-volume ratio is considerably higher than in large axolotls. This ratio must unquestionably determine (other conditions being equal) whether or not the larvae can obtain enough oxygen through the skin and pharynx to satisfy the increased oxygen demand during metamorphosis. This size relationship was discussed previously (Figge, 1929) in relation to the metamorphosis of lungless salamanders. These animals metamorphose when small, and are never very large. In the adult, the cutaneous and pharyngeal respiration seem to be sufficient. The pharynx and parts of the skin are supplied by the sixth aortic arch.

Helff removed the lungs of tadpoles and found that this decreased metabolism for a short time, indicating a functional larval lung. These were not essential to metamorphosis, because the integumentary respiration was adequate.

Garber (1930) removed the lungs from axolotls and produced metamorphosis by thyroid-feeding. The animals were at sea-level and kept in "aërated water." But even under the optimum conditions for metamorphosis, out of a total of five animals, one died before showing signs of metamorphosis, two died just after metamorphosis, and two survived metamorphosis. No difference was demonstrated between control and operated animals, as the feeding was not quantitative. There was also only one thyroid-fed control. The lung extirpation experiments are interesting, to say the least, but are not, as Garber claims, the equivalent of the ligation of the sixth aortic (pulmonary) arch, since this vessel has cutaneous and pharyngeal branches in addition to those going to the lung. He examined the adult animals to make certain that the lungs had been completely removed. It would have been more interesting to know the fate of the sixth arch in these lungless animals; but this was, unfortunately, not mentioned. It would seem probable that the sixth aortic arch

remained and that its skin and pharyngeal branches underwent hypertrophy to supply an increased amount of blood to the pharynx and skin, as it does in the normal lungless forms.

SUMMARY

EXPERIMENT I

The effect of ligation of the pulmonary arch on metamorphosis varies with the environment. In an environment that is optimum in regard to the availability of oxygen (temperature, 18°–20° C.; sea-level pressure, 76 cm.; running water) the ligated animals may metamorphose and survive as adults. The effect of the ligation in such an environment is evident only in the retardation of the onset of metamorphosis in general and gill reduction in particular.

EXPERIMENT II

By carefully controlling the environmental conditions, especially with regard to temperature, it was possible to maintain an environment in which non-ligated animals metamorphosed slowly. Ligated animals in the same environment remained larval for over a year, indefinitely, or 400 days longer than non-ligated. This indicates that the animals in which the pulmonary arch has been ligated are less sensitive or have a higher threshold for metamorphic stimuli than the non-ligated animals.

EXPERIMENT III

a) By injecting thyroid extract (internal metamorphic stimulus), it is possible to produce metamorphosis in ligated animals even at 6°–10° C. The amount of thyroid extract necessary to produce any given change is, however, far greater than the amount necessary to produce the same change in a non-ligated animal (amounts stated in Table II).

b) It was not possible to produce metamorphosis in ligated animals at 60 cm. pressure and 6°–10° C. They died either by bleeding or when the gill filaments began to reduce. Non-ligated animals under the same conditions can be metamorphosed by injections of thyroid extract.

c) The injection of thyreo-activator, extracted from prehypophysis—a more normal, more easily controlled, stimulus than thyroid injections—proved sufficient to metamorphose non-ligated animals but produced only copious skin-shedding in the ligated animals.

GENERAL CONCLUSIONS

a) Metamorphosis may be produced in ligated animals only if they are kept in an environment of relatively high oxygen tension.

b) The absence of the sixth aortic (pulmonary) arch decreases the efficiency of respiration at points other than the gills. Ligated animals die easily and are unable to metamorphose in an environment low in oxygen tension (60 cm.).

c) The hypothesis that gill reduction is in some way related to the oxygen content of the blood sent to the gills is further substantiated.

d) This work is presented as additional evidence in favor of the theory that the absence of the ventral portion of the sixth aortic (pulmonary) arch and the spiral valve in the perennibranchiates is one of the factors responsible for the retention of gills in those forms.

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THE RESPONSE OF RANA CATESBEIANA LARVAE TO INJECTIONS OF ANTUITRIN S AND PHYONE[1]

(Ten figures)

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IT HAS been repeatedly demonstrated that the anterior lobe of the hypophysis controls the activity of the thyroid gland. The work of Allen (1916, 1917), Smith, (1916, 1919), and others has definitely shown that atrophy of the thyroid occurs following ablation of the hypophysis in amphibia. Conversely, the work of Allen (1920), Smith (1922), Uhlenhuth (1927*a*), Ingram (1928), and Adams (1931) in their work on Amphibia, and Loeb (1929) and Aron (1930) in work on the guinea pig, has demonstrated a hypertrophy and hyperplasia of the thyroid following hyperpituitarism induced by implantation or injection. In addition to the thyroid effect, the anterior lobe exerts its well-known effects both on general body growth and on the development of the gonads (Evans and Long, 1921, 1922). Evans and Long prepared a crude extract of the anterior lobe which produced a pronounced growth in rats when injected intraperitoneally. More recently, Van Dyke and Wallen-Lawrence (1930) have prepared a highly concentrated and purified extract which appears to be free of the sex principle when tested on rats. This extract is now being prepared commercially by the Wilson Laboratories. An anterior pituitary-like sex hormone has been demonstrated by Ascheim and Zondek (1928) to be present in the urine of pregnancy, and a highly purified and concentrated extract of this kind is being prepared by the Parke Davis & Company. This work was undertaken to determine primarily what effects injections of these two extracts would have upon the thyroid glands of anuran

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larvae and, in addition, to determine their effects upon body growth, as indicated by body and total measurements, and upon the development of the gonads.

MATERIAL AND METHODS

The 135 animals used for this experiment were normal *Rana catesbeiana* larvae, selected from a group of tadpoles taken from two different sources. Those taken from one pool were used for antuitrin S injections and for their controls, while those taken from the second pool were used for phyone injections and for their controls. In both cases, all animals were selected so that maximum variation did not exceed 5 mm. in body length and 10 mm. in total length. The animals used for the antuitrin S experiment showed hind limb buds of 3 mm. or less, while those used for the phyone experiment had no hind limb buds whatever.

Commercial preparations of both growth and sex principles were used. Antuitrin S was obtained from Parke Davis & Company, and was furnished in part by them, and phyone was secured from the Wilson Laboratories. Before use in this experiment both of these extracts were tested. Two immature female rats, 33 days of age, that were injected with 0.035 cc. of antuitrin S twice daily for 3 days showed a definite precocious sexual development over their controls. The injected animals were autopsied 100 hours after the first injection. The uteri were found to be hypertrophied, and a microscopic examination of the ovaries showed formation of corpora lutea, conditions not found in the controls. The phyone was injected into six adult female rats, in the ratio of 0.35 cc. per kilogram body weight each day for 3 days. In that time the injected animals showed an average total weight increase of 3 per cent over an equal number of controls. The potency of these extracts was therefore verified.

The tadpoles receiving antuitrin S injections were divided into three groups: two groups (1AS and 2AS) of fifteen each, and one group (3AS) of ten. The controls for these animals were divided into two groups; one (1BS) of fifteen and one (2BS) of ten. The animals were given daily intraperitoneal injections of 0.05 cc. for 10 days. One group (3AS) received daily injections of 0.1 cc. for 10 days. Measurements of body length and total length were taken at the beginning of the experiment and again at the end of the experiment.

Animals receiving phyone injections were grouped in a similar manner. Two groups (4AP and 5AP) of fifteen each received daily intraperitoneal injections of 0.05 cc. for 10 days. The controls for these were likewise separated into two groups of fifteen each (3BP and 4PB). Measurements were made in the same manner as in the case of the antuitrin-S-treated animals. One group of ten tadpoles were measured at the beginning of the experiment but were not measured at the end of 10 days, since injections were continued until they metamorphosed.

For examination of the thyroid glands, the tissues were fixed in Bouin's fluid and in Zenker-formol and were stained with iron haematoxylin and eosin, and with a modification of Mallory's triple stain. Sections were cut 5 μ to 7.5 μ in thickness. In order to determine the volume of the thyroids, the glands were serially sectioned at 20 μ, and camera lucida outlines were made at a magnification of $\times 150$. The area of these tracings was then calculated with a Leitz planimeter and the volumes computed. The determination of the number of mitotic figures in the glands was made with a compound microscope, using an oil immersion objective and a $5\times$ ocular, from serial sections cut 7.5 μ.

Each group was kept in a separate glass aquarium, measuring $10\times 12\times 10$ inches, which was planted with *Vallisneria* and aërated with compressed air necessitating but one change of water in the duration of the experiment. All animals were fed a desiccated food made of one part egg yolk, one part powdered alfalfa, and six parts flour.

RESULTS

Antuitrin S injections.—Antuitrin S, when injected into these larvae, produced no apparent changes. Measurements given in Table I show that neither body length nor total length undergo any change as compared with those of the controls. Groups 1AS and 2AS, which received 0.05 cc. of the extract daily, showed no more appreciable difference in variation in these measurements than did those of group 3AS, which received twice as much extract (0.1 cc. daily). That no indication of metamorphosis in the appearance of the animals occurred is evident from a comparison of Figures 1 and 2.

An examination of the gonads showed no observable changes from

the gonads of the controls. Considerable variation was found in the size of both experimental and control gonads, which did not warrant

TABLE I

BODY LENGTH AND TOTAL LENGTH MEASUREMENTS (MILLIMETERS) IN CONTROL AND ANTUITRIN-S-INJECTED TADPOLES

	Antuitrin-S-injected			Controls	
	1AS	2AS	3AS	1BS	2BS
Number of specimens	15	15	10	15	10
Average body length at beginning of experiment	27.6	28.6	30.1	30.3	29.0
Average body length at end of experiment	26.9	27.5	29.5	30.0	28.5
Average body length difference	− 0.7	− 1.1	− 0.6	− 0.3	− 0.5
Average total length at beginning of experiment	63.2	62.8	64.3	64.1	63.0
Average total length at end of experiment	62.8	62.1	63.9	64.0	63.1
Average total length difference	− 0.4	− 0.7	− 0.4	− 0.1	+ 0.1

TABLE II

BODY LENGTH AND TOTAL LENGTH MEASUREMENTS (MILLIMETERS) IN CONTROL AND PHYONE-INJECTED TADPOLES

	Phyone-injected			Controls	
	4AP	5AP	6AP	3BP	4BP
Number of specimens	15	15	10	15	15
Average body length at beginning of experiment	30.4	29.4	30.3	30.3	29.8
Average body length at end of experiment	32.5	32.5	.....	30.5	29.9
Average body length difference	+ 2.1	+ 3.1	.....	+ 0.2	+ 0.1
Average total length at beginning of experiment	66.1	65.6	66.0	66.1	65.6
Average total length at end of experiment	70.1	69.6	.....	66.1	65.5
Average total length difference	+ 4.0	+ 4.1	.....	+ 0.0	+ 0.1

the recording of these data. Microscopic study revealed no apparent change in size, number, or degree of development of the germ cells or of the interstitial tissue.

The thyroid glands of the injected animals showed the characteristic histological picture of a normal gland. Large round follicles were present, well filled with an acidophilic colloid which stained readily with eosin. Numerous small clear vacuoles were present in the periphery of these colloid masses (see Figs. 5, 6, and 8). The epithelial cells lining the follicles were definitely flattened and cuboidal in shape, with no discernable differentiation of the cytoplasm.

TABLE III

VOLUME OF THYROID GLANDS (IN CUBIC MILLIMETERS) IN ANTUITRIN-S-INJECTED, PHYONE-INJECTED, AND CONTROL *Rana catesbeiana* LARVAE

Specimens	Antuitrin-S Control	Antuitrin-S-injected	Phyone Control	Phyone-injected
1	0.139	0.105	0.051	0.445
2	0.115	0.132	0.053	0.478
3	0.113	0.099	0.085	0.435
4	0.097	0.108	0.048	0.363
5	0.114	0.114	0.052	0.385
Average	0.115	0.114	0.058	0.421
Percentage difference	−0.87		+625.86	

The limits of the clear cytoplasm could be seen only with difficulty, and granular material within it was practically non-apparent. In the thyroids of both the control and treated tadpoles the picture is one of inactivity.

With such a quiescent picture presented, it would not be expected that any hypertrophy of the thyroid occurred. The volumes of the thyroids of five injected animals and five controls were determined, and the results are given in Table III. No change in volume had occurred, and the slight variation shown in Table III is within experimental error.

Phyone injections.—Injections of 0.05 cc. of phyone daily, given intraperitoneally, for 10 days produced marked changes in body length and tail length. Table II shows an average increase in body

length of 2.6 mm., while the controls show an average increase of less than 0.2 mm. The total length shows even a greater increase—over 4.0 mm. for the injected animals, as compared with less than 0.1 mm. for the controls. It is evident from these measurements that phyone, the growth principle from beef pituitaries, produces a marked growth effect when injected into *Rana catesbeiana* larvae.

The gonads of the phyone-treated animals are apparently not affected by this treatment, since both a macroscopic and microscopic examination revealed no observable changes from the condition found in the controls.

As may be judged from a study of Figures 7, 8, 9, and 10, the thyroids of phyone-treated animals show a striking change from those of the controls. The follicles have lost their roundness and appear to have collapsed, with only traces of the lumen remaining, and in addition appear to be completely emptied of their chromophilic colloid. Only in one case was there evidence of the stored colloid remaining, and then but a minute quantity in only one follicle in the entire gland. The follicular epithelium has likewise undergone considerable modification. The flat cells which make up the walls of the follicles of normal glands have become elongated to a columnar form (Figs. 9 and 10); and the nuclei appear to be enlarged and located, for the most part, at the basal ends of the cells, though on more careful examination, occasional cells may be found with the nuclei at the apical end. Though the significance of this placement of the nuclei has not been determined in this work, it is possible that they may represent the Bensley type of cell containing large amounts of colloid. The picture is readily recognizable as one of intense activity.

A striking difference in the volume of the thyroid glands of phyone-treated and control animals is shown in Table III. The average volume of the thyroid glands of five controls is seen to be 0.058 cc., while for an equal number of treated animals the average volume is 0.421 cc. These figures represent a volume increase of approximately 626 per cent. The hypertrophy displayed by these glands appears to be due to at least two factors, namely, (1) a growth in size and an increase in number of the follicular epithelial cells and (2) an increase in the vascularity of the entire gland. The growth in size of the follicular cells is made evident by reference to Figures 9 and 10, which

are camera lucida drawings of a small follicle from a control and a treated animal drawn to the same magnification. With the number of mitotic figures taken to be an indication of an increase in the number of follicular cells, Table IV demonstrates the proliferative activity of these cells. In serial sections cut 7.5 μ, the average number of mitotic figures per whole control gland was found to be 30.5, while a similar count of treated animals was found to be 395 mitotic figures per whole gland. This demonstrates an increase in proliferation in the glands of treated animals of approximately 1,295 per cent.

TABLE IV

NUMBER OF MITOTIC FIGURES IN THYROID GLANDS OF PHYONE-INJECTED AND CONTROL *Rana catesbeiana* LARVAE

Specimens	Phyone-injected	Control
1	445	14
2	364	32
3	387	16
4	451	26
5	328	39
Total	1,975	127
Average	395.0	30.5
Percentage increase	1,295	0

Equally important in bringing about the volume increase as shown in this work is the striking increase in vascularization. Only a perfunctory examination of sectioned material is necessary to establish this fact, which is indicated by a comparison of Figures 7 and 8.

Because of this evidence of hyperactivity on the part of the thyroid gland, it was assumed that precocious metamorphosis would be induced by continued treatment with phyone. One group of tadpoles (6AP) was injected with 0.05 cc. phyone daily for 28 days. At that time metamorphosis was clearly indicated, as may be seen from a comparison of Figures 3 and 4, showing a great increase in hind-leg length, the appearance in some cases of one fore limb, and a change in shape of the body approaching that of adult frogs.

DISCUSSION

It is apparent from the results obtained in this investigation that the anterior pituitary-like hormone extracted from urine of pregnancy exerts no growth effect on *Rana catesbeiana* larvae, while the growth hormone from the anterior lobe of beef pituitaries prepared by the method of Van Dyke and Wallen-Lawrence does. The fact that measurements given in Table I show a slight shrinkage in antuitrin-S-injected animals is not significant, since the controls show a similar decrease in size, this difference being due, in all probability, not to a toxic effect of the extract but to other factors such as lack of sufficient food, or more probably to errors in measurement. Phyone, on the other hand, produces a definite increase in size, which is in accord with the work of Smith and Smith (1923), who demonstrated that intraperitoneal injections of *pars anterior* extracts produced an increase in size in both hypophysectomized and normal tadpoles of *Rana boyeli*, and with the work of Adams (1932), who obtained a positive growth effect in *Triturus viridescens* by injections of phyone.

The failure of the gonads to show any observable changes as a result of injections of either of these two extracts is not quite clear. Antuitrin S has been shown to be effective in most laboratory animals in the induction of ovulation and precocious sexual maturity, but is without effect on the anuran larvae used in this work. Although phyone has been demonstrated to be free of the sex principle when tested on rats, Schockaert (1931) has produced an enlargement of the testes in ducks; and Hertz, Hellbaum, and Hisaw (1932) have produced follicular enlargement and luteinization of the ovaries of rabbits by phyone injection, which probably indicates that this preparation is not entirely free of a gonadotropic hormone. Adams (1932) reports a sex effect on adult *Triturus viridescens* by injections of phyone, while our work shows none. This difference in results should not be stressed, as it must be remembered that Adams' work was carried on with adult urodeles, while our work was done on larval anura. It is entirely possible that this apparent discrepancy may be due either to a difference in ability to respond among urodeles and anura or to the difference in ages of the animals used, in that the

PLATE I

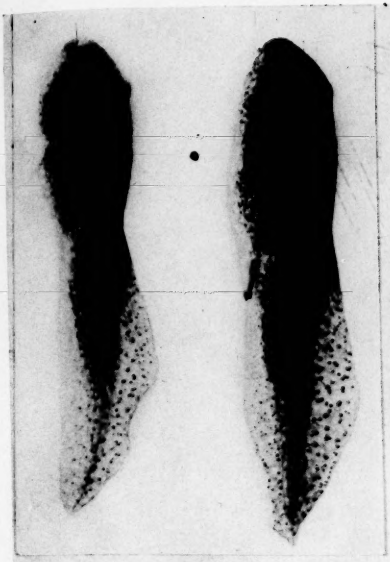
FIG. 1.—Photograph of two *Rana catesbeiana* larvae from control group for antuitrin S injections. ×1.2.

FIG. 2.—Photograph of two *R. catesbeiana* larvae after ten daily injections of 0.05 cc. antuitrin S. × 1.2.

FIG. 3.—Photograph of two *R. catesbeiana* larvae from control group for phyone injections. Natural size.

FIG. 4.—Photograph of two *R. catesbeiana* larvae after twenty-eight daily injections of 0.05 cc. phyone. Natural size.

PLATE I



G. 1

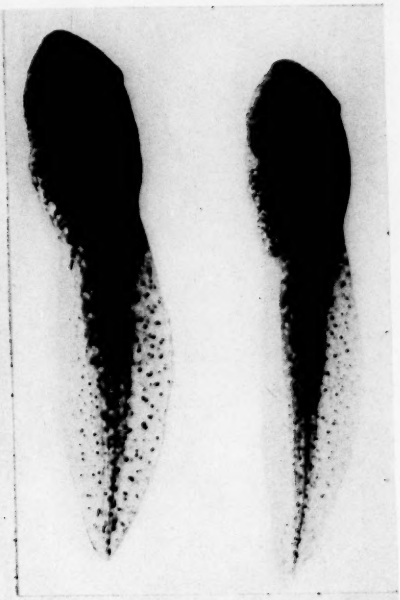


FIG. 2

G. 3

FIG. 4

gonads of the larvae may not have reached a sufficiently advanced stage of development to respond to the hormones.

Although Bourge (1930) reports a thyroid picture in rats corresponding to increased secretory activity as a result of injections of pregnancy urine, the work of Loeb (1932) does not confirm his results. Loeb found that injections of pregnancy urine do not affect the thyroid glands of guinea pigs but do affect the ovaries, differing in this respect from anterior pituitary substance and extract administration. With respect to its effect on the thyroid, our results with antuitrin S are in accord with those of Loeb.

The histology of the thyroid gland in control and experimental animals follows closely the description given by Uhlenhuth (1927*a*) on the morphology and physiology of the thyroid gland of *Ambystoma opacum*. Adams (1931, 1932), who obtained hypertrophy of the thyroid in salamanders by both implantation and inoculation of anterior pituitary, and Ingram (1928), by implantation in anura, report results similar to those obtained in our work with phyone injections. This hypertrophy we find to be due to a stimulation of proliferative activity of the follicular epithelium, as is evident from the data recorded in Table IV, showing an increase of nearly 1,300 per cent in the number of mitotic figures present in the entire gland. Equally important in bringing about this increase in volume is the tremendous increase in the vascularity of the gland. In studying serial cross-sections of these hypertrophied thyroids, it is not uncommon to find that half the total area of the section, and sometimes more, is made up of corpuscles lying in blood channels. This work has also shown that injections of phyone cause an emptying of the follicles of their stored colloid. It appears, then, that the thyroid stimulation present in the extracts used brings about a release of the stored colloid resulting in the emptying of the follicles.

Because this work has demonstrated thyroid hypertrophy as a result of phyone injections in these larvae, we do not infer that the growth hormone contained in this extract is necessarily a thyroid activator as well. It is interesting to note that both Rumph and Smith (1926), and Smith and Dortzbach (1931) have demonstrated that in the anterior lobe of the fetal pig the growth principle is detectable in a younger fetus than is the gonad-stimulating principle.

PLATE II

FIG. 5.—Photograph of a small region of a transverse section through the thyroid gland from a tadpole after ten daily injections of 0.05 cc. antuitrin S. × 335.

FIG. 6.—Photograph of a small region of a transverse section through the thyroid gland from control animal for antuitrin S. injections × 335.

FIG. 7.—Photograph of a small region of a transverse section through the thyroid gland from a tadpole after ten daily injections of 0.05 cc. phyone. × 335.

FIG. 8.—Photograph of a small region of a transverse section through the thyroid gland from a control animal for phyone injections. × 335.

FIG. 9.—Camera lucida drawing of a single small follicle from the thyroid of a tadpole after ten daily injections of 0.05 cc. phyone. × 1,000.

FIG. 10.—Camera lucida drawing of a single small follicle from the thyroid of a control animal. × 1,000.

PLATE II

FIG. 5

FIG. 6

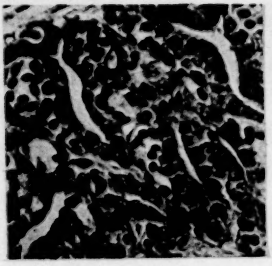


FIG. 7

FIG. 8

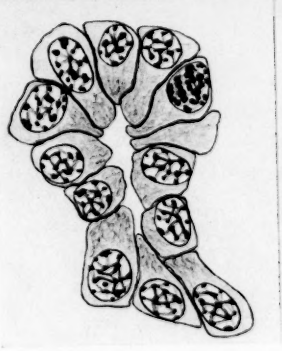


FIG. 9

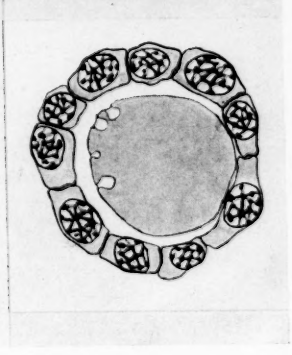


FIG. 10

They have also demonstrated that the thyroid hormone appears at the same time as the growth principle, which they do not consider surprising because of the close, well-established interrelationship between the two organs. On the other hand, in a recent preliminary paper, Anderson and Collip (1933) report having prepared an extract from the anterior lobe containing a thyreotropic factor which was obtained after the removal of the growth hormone and then later freed of prolactin. Their method of extraction is not given, and it is not stated whether their extract is free of a gonadotropic principle. At the same time, Riddle, Bates, and Dykshorn (1933) have just recently published a preliminary report on thyroid hypertrophy as a result of injections of the gonad-stimulating hormone, free from the growth hormone, which they have prepared from the pituitary. They conclude that "hyperplasia of the normally developed thyroid following pituitary administration is a specific response to the gonad-stimulating hormone, or to another pituitary derivative having very similar solubilities." Anderson and Collip, as well as Riddle, Bates, and Dykshorn, have therefore demonstrated that thyroid stimulation can be obtained from growth-free extracts, while Rumph and Smith, and Smith and Dortzbach have demonstrated a thyroid hormone to be present in the thyroids of fetal pigs at the same time that the growth principle is present in the pituitaries, which is at a considerably younger stage than that in which the maturity principle appears. In other words, the former groups of workers have demonstrated thyroid activation to be free of the growth hormone, while the latter workers present evidence which may indicate that thyroid activation is probably separate from the maturity hormone. It is apparent, then, that there still exists insufficient evidence to determine whether thyroid activation is a response to the growth hormone, the gonad-stimulating hormone, or to a separate and specific thyroid hormone.

SUMMARY

1. Injections of antuitrin S have no effect upon the growth of *Rana catesbeiana* larvae, while injections of phyone bring about an increase in body measurements.

2. Neither antuitrin S nor phyone injections produce any observable changes in the gonads of these larvae.

3. Antuitrin S injections do not affect the thyroid glands of *Rana catesbeiana* larvae, while injections of phyone bring about marked hypertrophy and hyperplasia.

4. The hypertrophied thyroids of phyone-treated tadpoles are greatly increased in volume because of an increase in size and number of the cells of the follicular epithelium, and because of an increase in the vascularization of the entire gland.

5. Injections of phyone bring about a release of the stored colloid within the follicles of the thyroid gland.

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VARIATIONS IN GROWTH-PROMOTING POWER FOR PLANARIAN WORMS OF ADULT AND EMBRYONIC TISSUES[1]

(Eleven figures)

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FEW investigators have used whole raw tissues in their nutrition studies. Where it has been done, it has led to some promising results. For instance, Titcomb, *et al.* (1928) failed to rear trout upon purified rations, but when these were supplemented with about one-fourth of their weight of raw meat, growth could be obtained.

In the tissue culture work Baker and Carrel (1928*a*) found that pepsin hydrolytic products of pure proteins are deficient in certain substances and do not meet the entire nutritive requirement of the cells for an unlimited period of time. They found that some supplementary nutritive substances are present in fresh embryonic heart tissue. Baker and Carrel (1928*b*) reported that when they used pepsin digests of calf liver and anterior lobe of the pituitary as media for sarcomatous fibroblasts of the rat, the colonies increased in volume to as great an extent as they did when chick embyronic juice was used.

Different animal tissues obviously differ in the amount of growth-promoting substances they contain. Titcomb, *et al.* (1928) found that beef liver supplemented with dry skim milk is superior to any other combination as a diet for trout. Raw liver (McCay, Dilley, Crowell, 1928) in an amount as small as 5 per cent, produced a marked acceleration in growth. Raw spleen (McCay, *et al.*, 1930) proved moderately satisfactory as a supplement for some of their diets. Good growth was obtained with the trout fed on lung alone (Titcomb *et al.*, 1929).

Osborne and Mendel (1917) found that young rats failed to grow on a diet of meat powder, starch, fats, and salt mixture. They found

[1] Aided by a grant from the Research Council of the University of Oregon.

that in using dried pig liver instead of meat powder, the growth of the rats was much greater than normal. Thus there is a great contrast between liver and muscle products as regards their growth-promoting power.

Osborne and Mendel (1918) also found that pig heart, kidney, and brain tissue produced excellent growths in the rats.

In one of the early investigations in tissue culture, Carrel (1913) used extracts of chicken embryos, spleen, kidney, leucocytes, muscle, Rous sarcoma, and thyroid gland. They were added to the plasma medium of the tissue cells, and the extent of growth was measured. All the tissue extracts were found to activate the growth of connective tissue but the degree of activation varied much. Embryonic tissue juice was the most growth-promoting. Extracts of adult spleen, Rous sarcoma, and leucocytes were almost as growth-promoting as that of chicken embryos. Kidney and heart extracts were less active. Muscle and thyroid extracts caused some growth, but muscle was less growth-promoting than thyroid.

Since few workers have investigated the growth-promoting power of native tissues, the following research was undertaken in this field. The planarian worm[2] was selected as the test animal, as Wulzen (1923 and 1927) has shown that these worms exhibit measurable differences in growth depending upon the tissue fed. She took measurements only at the beginning and the end of the experiments. But it was considered desirable to investigate the growth cycle of the worms in greater detail.

In order to do this, several series of experiments were carried on, using raw tissues of the rabbit, chicken, guinea pig, and beef pituitary as diets. The following method was used: Each tissue used in the series of experiments was fed to a group of thirty newly regenerated worms, each worm 8 mm. in length. A generous amount of food was placed in the dish with the worms. Four or more hours later the excess food was removed and the water changed. There was no difficulty in getting them to eat any of the tissues used. Growth in length of the entire experimental group was taken as the criterion for comparison. The worms were measured by being placed in a Petri dish on a glass plate under which was a polar co-ordinate paper.

[2] Probably *Planaria agilis*.

They were made to glide by placing a light at one side, and, being negatively heliotropic, they traveled in the opposite direction. In order to get an accurate measurement they must be gliding smoothly. With practice one can tell whether the worms are fully extended or somewhat contracted.

The worms were fed their respective diets twice each week. If any of the worms became abnormal or underwent fission during the experiment they were removed. In order to construct the growth curves the growth of the remaining worms was used as a basis for calculating by simple proportion the growth of the original 30 worms. Seldom did the number get below 24, and in these cases it was due either to very rapid fission, as on a diet of spleen, or to a diseased condition for which the diet was responsible. Before each feeding the worms were kept in an incubator at the constant temperature of 22° C. Sterilized finger bowls were used for the experimental groups. The worms were kept in boiled river water which was changed at least once between feeding days.

Details on 3 typical experimental series follow. In these, adolescent rabbit, embryo chick, and older chicken tissues were used as experimental diets. Details of the other series are omitted, but growth curves from these series are shown in the final compilation. A total of 1,410 worms was used in the experiments reported in this paper.

SERIES I

The following tissues from adolescent rabbits were fed to a series of 6 experimental groups of worms for a period of 6 weeks: liver, skeletal muscle, heart, lung, spleen, and pancreas. All the tissues in this series were fed in a chunk, while in the remaining series all the tissues were finely ground. The resulting growths are plotted in Figure 1.

The worms fed on liver and spleen totaled practically the same growth until the fourth week, when the average final growth of the spleen-fed worms dropped as compared with the liver-fed worms. This drop can be explained by the fact that the spleen seemed to cause fission. At two and one-half weeks, two worms had undergone fission; at four weeks, six; at four and one-half weeks, thirteen; at five and one-half weeks, seven, leaving one worm which divided be-

fore the last measurement at six weeks. The drop in the curve of spleen-fed animals is undoubtedly associated with the fact that 13 worms divided.

Lung caused a good steady growth in the worms. Heart caused less growth. Pancreas and muscle were found to be poor growth-promoting tissues.

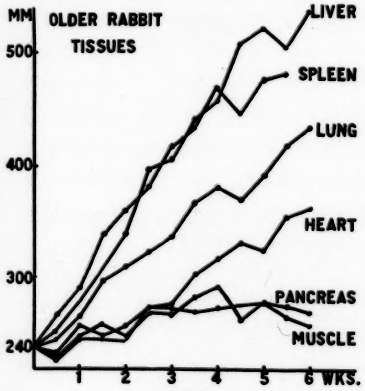

FIG. 1

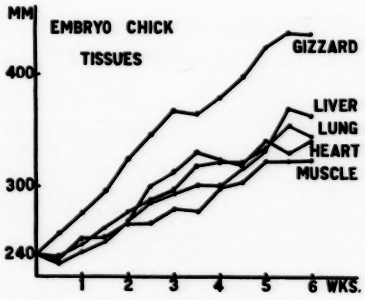

FIG. 2

FIG. 1.—Growth curves of 6 experimental groups, consisting of 30 worms each, fed on tissues of the adolescent rabbit.

FIG. 2.—Growth curves of 5 experimental groups of 30 worms each fed on tissues from embryo chicks varying in age from 1 to 18 days.

As to fission in the 6 weeks' period: 29 worms fed on spleen divided; 3 on liver; 2 each on lung, muscle, and heart; and none of those fed on pancreas. Thus while liver and spleen resemble each other closely in growth-activating effect, they differ greatly in their effect on fission.

SERIES V

Embryo chicken tissues were used in this series. Eggs were incubated in the laboratory, the length of incubation varied between two and three weeks. The tissues from two or three chicks were found to be sufficient. The following were used: heart, lung, liver, muscle, and gizzard. The growths for a six weeks' period are plotted in Figure 2.

In this series, the group of worms fed on gizzard showed the greatest growth. It is to be noted in the preceding series and those following that liver-fed worms grow exceedingly well. Liver has been found to be one of the best growth-promoting tissues. In this series, however, liver caused less growth in the worms than did heart and lung up to the fifth week, and at six weeks ended with only slightly greater growth than these two tissues. The growth of the muscle-fed worms, as will be noted later, is greater than usual.

SERIES VI

Tissues from non-adult chickens, 2–4 months of age, were used in this series of ten groups of worms. The following tissues were fed: heart, lung, liver, blood, muscle, pancreas, kidney, spleen, thymus, and gizzard.

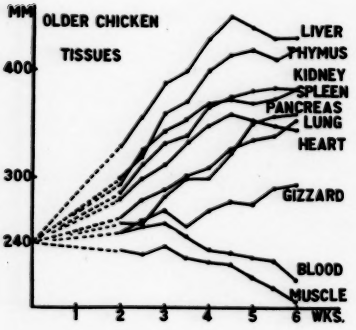

FIG. 3.—Growth curves of 10 experimental groups of 30 worms each fed on tissues of non-adult chickens, varying in age from two to four months. (The term "older" is used as compared with embryo tissues in Figure 2.) In order to clarify the chart, the first two weeks' growth is shown in dotted lines, and does not represent the details of growth.

The results appear in Figure 3. As shown here, liver-fed worms showed the greatest growth in the series. Thymus-fed worms were second. Worms fed on kidney and spleen showed very good growth. They agreed with each other closely in rate of growth.

The result of pancreas feeding is especially noteworthy. The growth of the worm fed on pancreas is fairly good, resulting in a slightly greater final length than that attained by worms fed on lung and heart. Pancreas, from other sources than the chicken, has been found to be a poor growth-promoting tissue. In fact, it has been found to contain a growth inhibitor (Wulzen and Bahrs, 1928). This is true of rabbit, beef, and guinea-pig pancreas.

Chicken pancreas appear to have a dissolving effect on some of the worms. They split lengthwise shortly after eating, leaving a

finely divided remnant of the former worm. At the end of the first two and one-half weeks of the experiment, 10 worms had gone to pieces. The remaining worms, which were able to withstand this effect, grew well. The explanation of this unusual action is not known.

Gizzard-fed worms showed poor growth while the worms fed on blood and muscle showed even a negative growth.

In order of growth-promoting effect of the older chicken tissues in regard to planarian growth, then, we have liver > thymus > kidney and spleen > pancreas, lung and heart > gizzard > blood and muscle. The term older is used in contrast to the embryonic tissues used in Series V. They are really adolescent chicken tissues.

DISCUSSION

From the series of experiments detailed above, and some additional ones, a number of comparisons are in order, namely, the contrast between the growth curves produced when using rabbit tissues and those produced when using chicken tissues, the comparison of the old and young tissues in both the rabbit and the chicken in regard to planarian growth, and the comparison of the growths of the worms fed on the same tissues at different times to see whether or not the results could be duplicated. Also we might classify the various tissues used in order of their growth-promoting effect on planarian worms and note the general characteristics of the growth curves with special reference to rate of growth.

A. GROWTH OF WORMS FED ON TISSUES OF THE RABBIT CONTRASTED WITH GROWTH ATTAINED ON CHICKEN TISSUES

Comparison of the growth produced in Planaria fed the same tissues but from different animals, i.e., the rabbit and chicken are shown in Table I and in Figures 4 and 5.

In the cases of worms fed on spleen, kidney, and muscle shown in Figure 4, curve sets 1, 2, and 5, and liver and lung shown in Figure 5, curve sets 3 and 4, the rabbit tissues produced greater growths in the worms than did the corresponding chicken tissues.

In the case of worms fed on thymus, Figure 5, curve set 6, and heart, Figure 4, curve set 7, the final growths did not show a significant difference. Pancreas, Figure 5, curve set 8, was the only

TABLE I

COMPARISON OF GROWTH PROMOTING POWER OF OLDER RABBIT AND OLDER CHICKEN TISSUES

Tissue Used	Chart and Curve Set	Growth-Promoting Power of Rabbit > or < Chicken Tissue	Terminal Difference in Length of Experimental Groups in Mm.	Difference in Mean Length in Mm.	No. of Times Difference Is Greater than the Probable Error
Spleen	IV (1)	Rabbit > Chicken	217	7.230 ± .293	24.6
Kidney	IV (2)	" > "	146	4.890 ± .310	15.8
Liver	V (3)	" > "	106	3.568 ± .285	13.0
Lung	V (4)	" > "	80	2.757 ± .202	13.0
Muscle	IV (5)	" > "	73	3.465 ± .239	14.0
Thymus	V (6)	About same up to $4\frac{1}{2}$ weeks Then Rabbit > Chicken	42	0.286 ± .292	.....
Heart	IV (7)	Chicken > Rabbit at 3 weeks	35		
		Rabbit > Chicken at 6 weeks	16	0.551 ± .167	3.3
Pancreas	V (8)	Chicken > Rabbit	89	2.967 ± .216	13.0

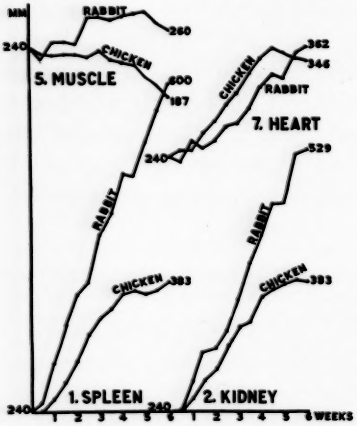

FIG. 4

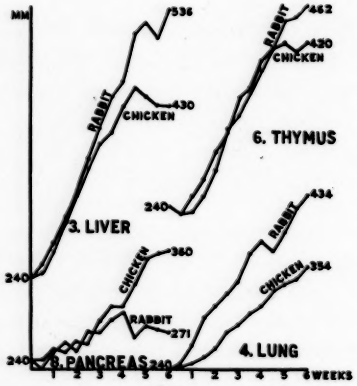

FIG. 5

FIG. 4.—Growth curves from experimental groups of 30 worms each fed on tissues of the chicken and rabbit, plotted together for comparison. Includes spleen, kidney, muscle, and heart. Figures indicate length in mm. of experimental groups at the conclusion of the experiments. Each group measured 240 mm. at the beginning.

FIG. 5.—Growth curves from experimental groups of 30 worms each fed on tissues of the chicken and rabbit, plotted together for comparison. Includes liver, lung, thymus, and pancreas. Figures indicate length in mm. of experimental groups at the conclusion of the experiments. Each group measured 240 mm. at the beginning.

tissue in the chicken which produced a greater growth in the worms than did the corresponding rabbit tissue.

As can be seen in Table I, the mean differences in the length of the worms are, in all cases except thymus and heart, 13 or more times their probable errors. Thus the differences are statistically significant.

In comparing the growths of the worms at six weeks, then, in five of the tissues fed, rabbit > chicken; in two of the tissues, the growth was about the same; and in one tissue only chicken > rabbit. In general we might conclude that rabbit tissues contain more of the substances necessary for planarian growth, or perhaps less harmful substances preventing growth than do the chicken tissues.

When we arrange the tissues of the chicken and rabbit in order of their effect on planarian growth, we find them grouped as follows:

TISSUES ARRANGED IN ORDER OF DECREASING GROWTH-PROMOTING POWER FOR PLANARIAN WORMS

Rabbit	Chicken
Spleen	Liver
Kidney	Thymus
Liver	Kidney
Thymus	Spleen
Lung	Pancreas
Heart	Lung
Submaxillary Gland	Heart
Pancreas	Gizzard
Muscle	Blood
	Muscle

There is a similarity in the general order of the tissues. Muscle is at the end of the list in both cases. The general relation of liver, thymus, lung, and heart is the same. The exceptions are liver and thymus, which take precedence over kidney and spleen in case of the chicken tissues; also the position of pancreas in chicken tissues as opposed to that in rabbit.

B. COMPARISON OF OLD AND YOUNG TISSUES

In their tissue culture work Carrel and Ebling (1922) found that the rate of multiplication of fibroblasts and the duration of their life in vitro varied in inverse ratio to the age of the animals from which the plasma or the serum was taken. Growth was more abundant in

the plasma of the younger than in that of the older animals. Bahrs (1931) feed digestive mucosa of different parts of the digestive tract of both young rabbits (2–3 months) and rabbits over a year old to planarian worms and obtained better growth from those worms fed on the younger tissues than those fed on the older.

In order to ascertain whether in these experiments the growth-promoting power of the tissues fed varied with the age of the animals producing the tissues, the following comparisons were made.

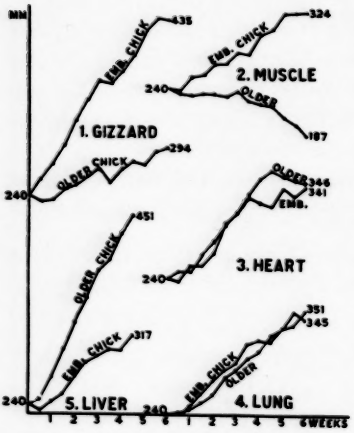

FIG. 6.—Comparison of the growth curves of experimental groups of worms fed on embryo chicken tissues and those fed on older chicken tissues (2–4 months old). Figures indicate length in mm. of experimental groups at the conclusion of the experiments. At the beginning each group measured 240 mm.

1. *Chicken tissues.*—Growth curves of worms fed on tissues from non-adult chickens, 2–4 months old, are compared with the curves of worms fed on tissues from embryo chicks, 2–3 weeks old, in Figure 6. It will be noted that liver, gizzard, and muscle, curve sets 5, 1, and 2, produce striking differences. Heart and lung of the different aged animals, curve sets 3 and 4, produced about the same respective growths in the worms.

As shown by the curves, gizzard and muscle of the embryo chick produced greater growths in the worms than those same tissues of the older chicken. Adult chicken liver, on the other hand, produced greater growth in the worms than did the embryo chick liver. There is a distinct difference in the appearance of the two: the older chicken liver had a deep red color, characteristic of liver, while the embryo chick liver had a yellowish appearance. That there is a difference in the growth substance seems quite certain.

That the differences noted in Figure 6 are significant statistically is shown in Table II. Comparison of the growth on liver is made at

$4\frac{1}{2}$ weeks instead of at 6 weeks as with the others because of disintegration that occurred in the worms fed on older chicken liver.

It would appear, then, that, in the embryo, storage of the growth-promoting substance was chiefly in the gizzard and muscle and to a lesser degree in the liver. In the older chicken the liver contained the growth substance in large amounts, while the muscle and gizzard lacked it, at least in amounts compared to that which they had in the embryo tissue.

The consistency of the tissue might play a part. The older chicken muscle and gizzard are quite fibrous and hard, while the embryo ones are much less so. However, all the tissues were ground, thereby exposing innumerable broken cells for the worms to devour. It is very doubtful if consistency of the tissues could account for the entire difference in the resulting growth.

TABLE II

COMPARISON OF EMBRYONIC AND OLDER CHICKEN TISSUES AS REGARDS PLANARIAN GROWTH

Tissue Used	Growth-Promoting Power of Emb. >or< Older Chicken Tissue	Terminal Difference of Experimental Groups in Mm.	Difference in Mean Length in Mm.	No. of Times Difference Is Greater than the Probable Error
1. Gizzard	Emb. > Older	141	4.708 ± .245	19
2. Muscle	Emb. > Older	137	4.466 ± .161	27
3. Heart	Older > Emb.	5	0.156 ± .293	.....
4. Lung	Older > Emb.	9	0.363 ± .201	1.8
5. Liver	Older > Emb.	134	4.474 ± .237	14.6

2. *Rabbit tissues.*—When the growth of the worms fed on older rabbit tissues was compared with the growth of worms fed on corresponding tissues from young rabbits 1–18 days old, no significant difference was found to occur. This was found to be true for muscle, heart, liver, lung, and kidney.

In this case, embryo rabbits were not obtainable, and the older rabbits used were only in the adolescent stage. It is very probable that there is a difference in the tissues as to the age at which they possess their maximum amount of growth-promoting substances, but the difference in ages between these two groups was not sufficient to bring it out.

3. *Guinea pig.*—In order to compare the effect on the worms of truly embryonic tissues and adult tissues, six experimental groups of worms were fed twice each week for a period of four weeks on guinea-pig tissues. Pregnant guinea pigs, which were practically at full term, furnished the adult tissues. Well-developed embryos taken from the uterus of the guinea furnished the embryonic tissues. Liver, heart, and lung were the tissues used. The growth of the worms fed on these tissues is shown in Figure 7.

Lung, curve set 2, of the embryo and adult guinea pig, caused practically identical growths in the worms. Heart, curve set 3, however, of the embryo guinea caused a significantly greater growth in the worms than did the adult guinea-pig heart. There was a mean difference of $2.267 \pm .162$ mm. (difference 14 times probable error).

Liver, curve set 1, from both the embryo and adult guineas, caused remarkable growth in the worms. The growth was practically the same up to three weeks. At four weeks the worms fed on liver from the embryos averaged a greater length than did worms fed on liver of the adult. However, toward the last of the experiment, the worms fed on the embryo liver underwent fission to a marked degree, leaving at the end of the experimental period only 4 worms, so this difference cannot be stressed.

Attention should be called to the following contrast. In the embryo guinea pig that had been receiving constant nourishment from the pregnant mother, the liver contained a great store of the growth-promoting substances in amounts equal to that contained in the mother's liver.

However, in the case of the developing embryo chick, dependent on the stored yolk of the egg, the liver lacked the growth-promoting substances.

C. DUPLICATING RESULTS

Whether or not the growth curves resulting from the different experiments can be duplicated is an important point. Among the tissues that were used more than once were adult rabbit liver, lung, and spleen. These curves are plotted in Figure 8. Thus the two lung curves resemble each other greatly and for all practical purposes we can say the results were the same.

In case of spleen, the curves were almost identical up to four

weeks. The great difference occurring after this time was due to fission in Series I. This brings in a factor which does not allow a true comparison. The three liver tissues have the greatest range. In spite of this it seems quite evident that when the variability as to the age, physical conditions, etc., in the animals used to supply the tissues are taken into consideration, we can say that the growth curves of the worms agree remarkably well.

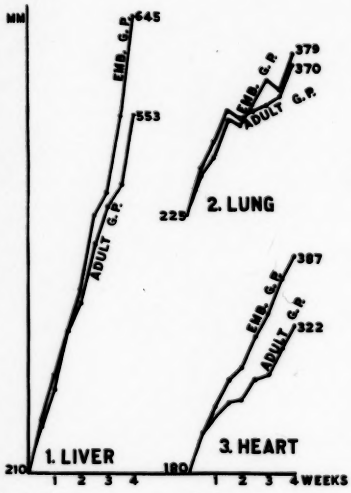

FIG. 7

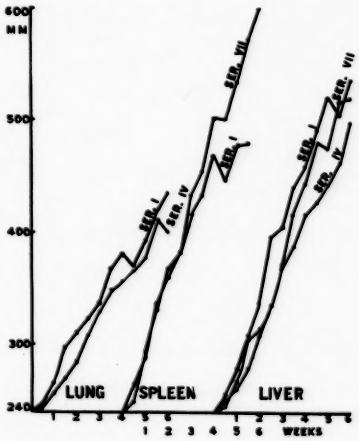

FIG. 8

FIG. 7.—Comparison of the growth curves of experimental groups of worms fed on adult guinea-pig tissues and those fed on embryo guinea-pig tissues.

FIG. 8.—Comparison of growth curves of experimental groups of worms fed on the same tissues in more than one series to see whether or not the results could be duplicated.

D. GENERAL RELATION OF THE RAW TISSUES USED IN THE SERIES OF EXPERIMENTS

Certain of the growth curves are plotted together in Figure 9. In selecting the curves to be plotted, this method was used. When the tissues of rabbit and chicken, old or young, differed in their activation of growth in the worms, all were plotted. If, on the other hand, they were approximately the same, only one was plotted to represent all the respective tissues. For example, heart tissue was practically

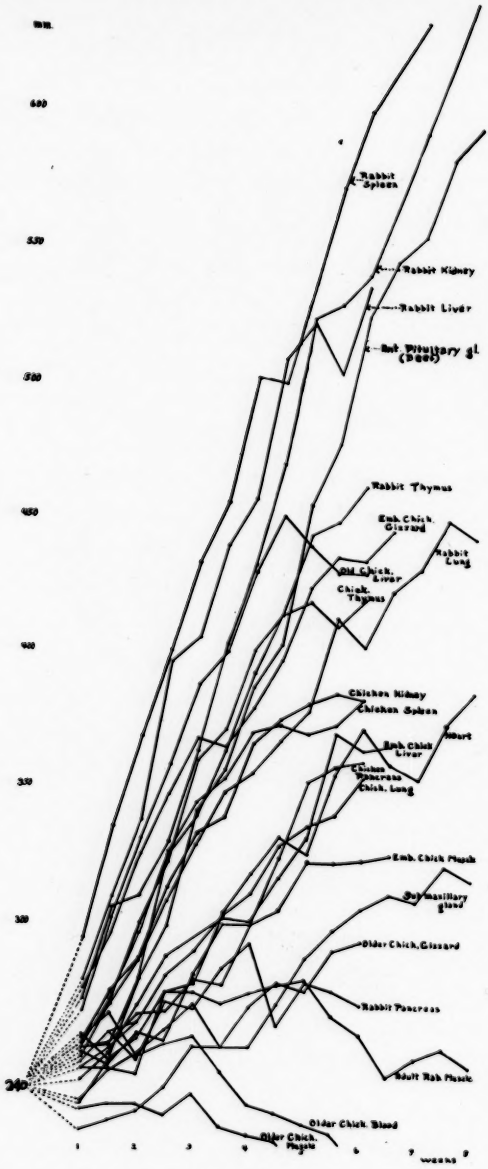

FIG. 9.—Compilation of some of the different growth curves taken from the series of experiments using raw tissues. Reading the labels from the bottom up gives the tissues in order of increasing growth-promoting power.

the same for the older and embryo chicken, as well as for the older and younger rabbit. Thus only one curve was shown on the chart. Also rabbit lung represents the growth of the worms fed on both the younger and older rabbit tissues, as the growth of these two was almost identical.

In order to clarify the chart, the first week's growth on the different tissues is shown in dotted lines, and does not represent the details of growth. As can be seen from the chart, the various tissues fall to some extent into groups. In order to ascertain where the significant breaks in the grouping occur, Figure 9 was used as a basis for Table III, where the mean lengths of the worms, fed the various tissues, are shown. A mean difference four or more times the probable error is considered sufficiently significant to separate the groups. Group III has the greatest range. It is divided into three sub-groups.

In considering the general arrangement of the tissues shown in Figure 9 the question arises whether the varying growths of the worms are due to varying amounts of growth-promoting and growth-inhibiting substances in the tissues fed, or whether it is due in large part to the availability of the tissues for the worms. It would seem quite logical to suppose that a soft tissue, brain for instance, would be more easily ingested and would result in a greater growth than would a firmer tissue like muscle.

There seems, however, to be no positive correlation between softness of the tissue and increased growth. Beef pituitary is a hard, firm tissue but produces greater growth in the worms than does older chicken liver which is less firm. Heart is a very firm tissue, practically as firm as skeletal muscle, yet it produces greater growth than does embryo chick liver, pancreas, and submaxillary gland, all of which are much softer. In addition, spleen, kidney, lung, and skeletal muscle taken from different animals, rabbit and chicken, did not differ in consistency as far as one could detect, yet they did differ markedly in growth-promoting power.

With brain tissue we have an interesting situation. The gray matter of the brain produces good growth in the worms while the white matter produces poor growth. A group of 30 worms fed on gray matter of rabbit brain gained 62 mm. in $2\frac{1}{2}$ weeks while a corresponding group, fed on white matter of the brain, gained only 17

mm., or a difference of 43 mm. Surely consistency of the tissues could not play a part here.

TABLE III

TABULATION OF MEAN LENGTHS OF WORMS FED THE VARIOUS TISSUES SHOWN ON CHART IX

(The Grouping of the Various Tissues Is Shown.)

Group	Tissue Fed	Mean Length and Probable Error of Worms in Mm.	Difference in Mean Lengths between Groups and Subgroups in Mm. and Probable Error of Difference*	No. of Times Difference Is Greater than Probable Error
I.	Spleen—rabbit	20.000 ± .218		
			2.360 ± .341	6.9
II.	Kidney—rabbit	17.640 ± .261		
	Liver—rabbit	17.852 ± .201		
	Anterior pituitary—beef	17.172 ± .205		
			2.672 ± .283	9.4
III.	(*a*) Gizzard—embryo chick	14.500 ± .195		
	Liver—older chicken	14.294 ± .203		
	Thymus—chicken	14.000 ± .162		
	Lung—rabbit	13.393 ± .149		
			0.643 ± .221	2.9
	(*b*) Kidney—chicken	12.750 ± .166		
	Spleen—chicken	12.770 ± .194		
			0.699 ± .217	3.2
	(*c*) Heart—rabbit and chicken	12.071 ± .095		
	Liver—embryo chick	12.115 ± .171		
	Pancreas—chicken	12.000 ± .189		
	Lung—chicken	11.846 ± .148		
			1.166 ± .187	6.2
IV.	(*a*) Muscle—embryo chick	10.680 ± .113		
			0.488 ± .156	3.1
	(*b*) Submaxillary gland—rabbit	10.192 ± .082		
	Gizzard—older chick	9.792 ± .149		
			0.759 ± .182	4.2
V.	Pancreas—rabbit	9.033 ± .104		
	Muscle—rabbit	9.679 ± .166		
			2.804 ± .207	13.5
VI.	Blood—chicken	6.875 ± .123		
	Muscle—older chicken	6.214 ± .114		

*The figures show the differences in mean lengths and probable errors of the differences between the highest member of one group and the lowest member of the preceding group.

The pharynx of the planarian worm is a strong little structure and can suck in food substances from practically any tissue. This is shown by the fact that a small bit of any of the tissues we have fed,

no matter how hard, when placed in a stock pan of worms, will disappear, except for minute, white strands of connective tissue which are hard to find.

E. GENERAL CHARACTERISTICS OF THE GROWTH CURVES OF THE PLANARIAN WORM

Chaddock (1925) points out that in plotting curves on ordinary graph paper, the slopes should not be compared as they do not represent rates of increase or decrease. It is the *levels* on the curves which are comparable and not the slopes. In order to be able to compare the growth rates, semi-logarithm paper was used. Here spacings in the vertical direction represent ratios and in the horizontal direction, absolute amounts.

Series VIII is shown in Figure 10 plotted on semi-logarithm paper. The slope of the curve between any two points represents rate of increase, the same slope indicating the same rate anywhere in the diagram. Thus the comparative steepness of different portions of the same curve indicates comparative rates of increase or decrease.

The greatest increase in growth rate in all three curves during the experimental period came between one and three weeks. From here on the rate declined quite steadily. In the case of thymus and lung the decline was much greater than in kidney.

Titcomb, *et al.* (1928) showed that the rat, chicken, and pig have rapid rates of growth, while the trout has a much slower rate, and that the phenomenon of acceleration of normal growth is rather difficult to show in such rapidly growing animals as the rat. The trout, they point out, seems superior for inanition studies with its slow but regular growth. The nearer the rate of growth of any animal approaches the perpendicular, the more difficult it is to show an acceleration. According to these investigators, the trout seems to be the best experimental animal available at present for studies upon growth acceleration.

In order to compare the rate of growth of the worms with those of the trout, the rat, and the chicken, their respective growths up to seven weeks, as given by Titcomb, *et al.*, were plotted in Figure 11. The data on the pullets did not begin until they were two weeks old.

The growth of all except the worms is judged by differences in weight. The worms are represented by growth in length. The rate of growth of the worms resembles a great deal that of the trout, with

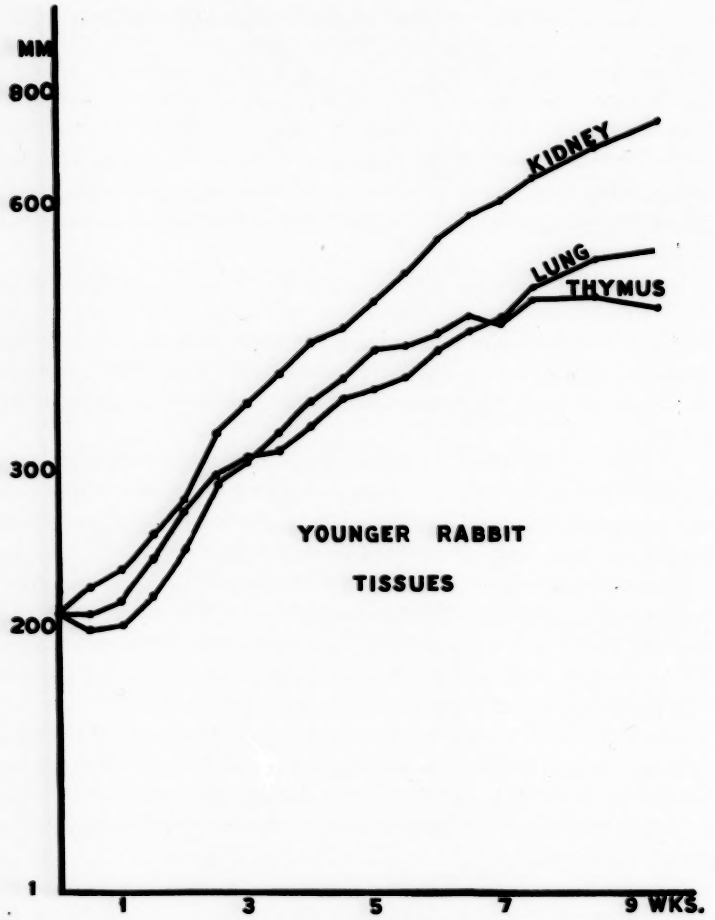

FIG. 10.—Growth curves of 3 of the experimental groups of worms of Series VIII, plotted on semi-logarithm paper. The comparative steepness of different portions of the same curve indicates comparative rates of increase or decrease.

a gradual, steady increasing growth. Therefore, the planaria can well be used along with the trout as a test animal for studies upon growth acceleration.

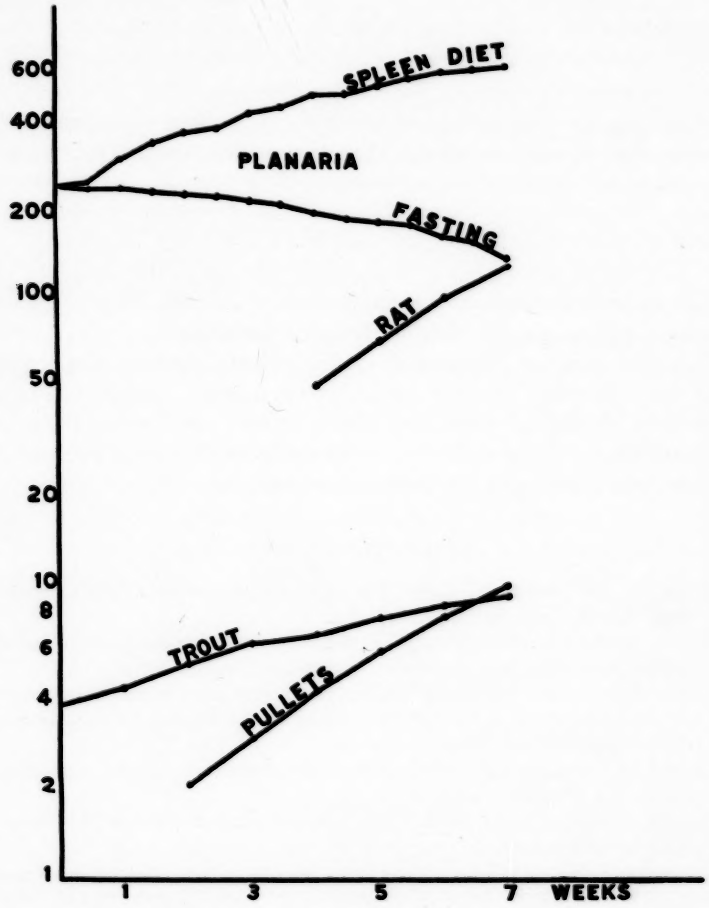

FIG. 11.—Growth curves of pullets, trout, rats, and planaria plotted in order to compare their relative rates of growth up to 7 weeks. The figures of all except the planaria are taken from Titcomb, *et al.* (1928).

SUMMARY

1. Chicken tissues were found, on the whole, to contain less of the substances necessary for planarian growth than did rabbit tissues. Pancreas of the chicken was the only tissue producing better growths in the worms than the corresponding rabbit tissue.

2. Embryo chick muscle and gizzard were found to contain more of the growth-promoting substances than the same tissues from older chickens. On the other hand, older chicken liver contained more than the embryo liver. Thus tissues differ in this factor, depending on the age of the animals.

3. Comparisons of young rabbit tissues (1–18 days) and adolescent rabbit tissues failed to bring out a significant difference.

4. Embryo-guinea pig liver and lung caused similar growths in the worms to those of the adult mother guinea. Embryo heart caused greater growth than did heart of the adult.

5. The greatest growth-rate during the experiments was found to occur between 1 and 3 weeks. From then on, the growth rate declined. Planaria have a slow, steady growth-rate, resembling that of the trout, as opposed to the more rapid growth-rates of the higher animals; for example, the chicken, rat, and pig.

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THE PHYSIOLOGICAL GRADIENTS OF HYDRA. III. RECONSTITUTION OF MASSES OF DISSOCIATED PIECES

(Thirty figures)

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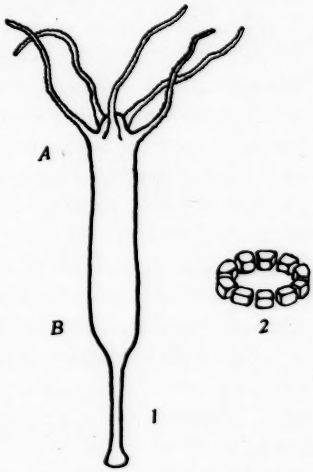
IN 1926 there appeared a report by Issajew on the reconstitution of dissociated parts of *Hydra*. In the experiments entire animals were cut into small pieces and massed together by placing the pieces in small depressions in paraffin in a minimum amount of water. New axes appeared, the loci of which were apparently determined by portions of the peristome as shown by the presence of the tentacles of the original animals which were present in these regions. The experiments here reported were made to determine, if possible, whether regions of the body other than the peristome of the animal would function as organization centers and if the differential of contact would have any effect on such organization and reconstitution.

METHOD AND MATERIAL

The animals used in these experiments were *Pelmatohydra oligactis* (Pallas) Schulze (Fig. 1) and came from a laboratory stock which was cultured from specimens collected in the lagoon in Jackson Park, Chicago, Illinois. The stock animals were kept in glass aquaria and fed regularly on *Daphnia*.

In contrast to the method used by Issajew (1926), who sectioned the entire animal, only the body regions of the animals in a minimal amount of water were placed on the concave surface of an inverted finger bowl. These were then sectioned transversely, resulting in a complete circle of body tissue, and these circles or rings were then divided into segments as small as possible. These small arcs of tissue were approximately 1 mm. long (Fig. 2). Some idea of the size of the pieces may be gathered from the protocol of a typical experiment.

"The distal halves of seven animals from which the peristome and tentacles were removed were cut into 110 pieces!" The cutting of these particles must be done as rapidly as possible since otherwise they will not cohere and grow together when centrifuged. This may be due to the formation of a film around them, but is more probably due to contraction, for they have a tendency to round off into small balls of tissue soon after cutting. This would place the ectoderm on the outside and thus prevent fusion, for according to Papenfuss (1933) the entoderm cells take the initiative in fusing apparently by the interlacing of the amoeboid processes of these cells. The optimum time limit for sectioning is from 15 to 20 minutes.



FIGS. 1–2.—Large non-budding *P. oligactis*. Sections taken from the body region *A–B* were cut as seen in Figure 2 and used in these experiments.

The pieces were counted as they were drawn into a small pipette the tip of which was drawn out to the diameter of a millimeter. They were then transferred to a conical centrifuge tube filled with filtered aerated culture water, placed in a centrifuge and centrifuged for $1\frac{1}{2}$ hours at a slow rate of speed, approximately 750 r.p.m. Thus all the pieces were thrown to the conical bottom of the tube and held in contact with each other.

Following the centrifuging, the pieces were allowed to remain in the tube for another hour and then the mass was removed into a stender dish filled with aerated culture water by inverting the tube with its open mouth below the surface of the water in the dish. Often the mass must be loosened around the edges, which seem to adhere to the tube. This was done by means of a needle. Care must be taken to exclude all air from the centrifuge tube when making the transfer since the change in tension on the mass when coming in contact with the air in the bubble often causes it to break up into small

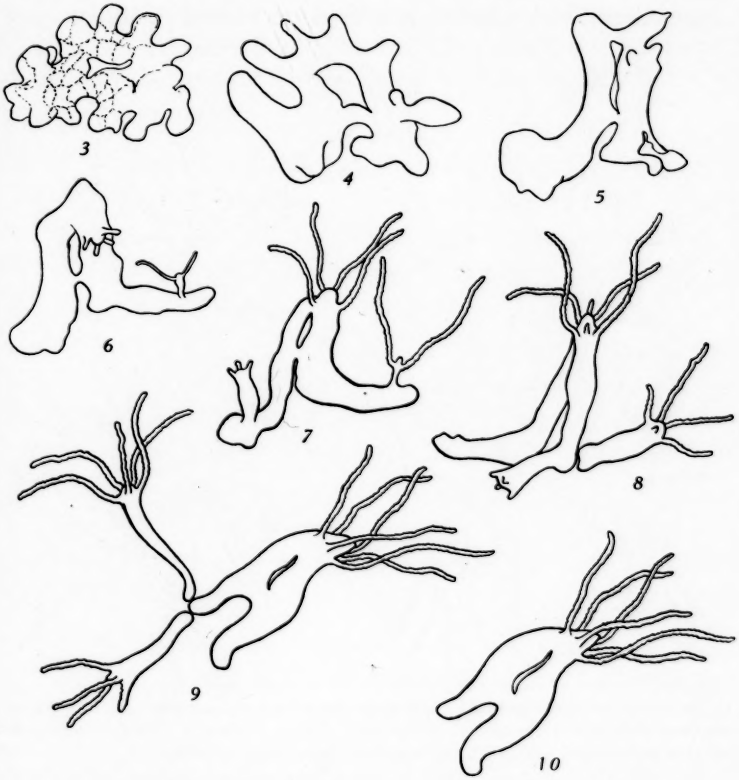
fragments. Very often in spite of these precautions the mass will break up into two or three smaller masses or lose some small fragments. The stender dishes were kept at room temperature and observations made daily. The appearance of the masses is shown in Figures 3–30. Attention is called to the fact that apparently 2 hours after centrifuging the external surface is covered by a layer of ectoderm. This phenomenon was also noted by Issajew (1926).

When the mass of pieces is first removed from the tube and examined under the microscope, it has somewhat the appearance of a mosaic with the outlines of many of the component pieces marked off by lighter areas (see Fig. 3). This appearance changes within a few hours and the mass becomes almost homogeneous. Small spaces may appear here and there in the mass. In very few instances are the masses free of these spaces. Sometimes the mass resembles a sort of tangled chain (Figs. 4–6).

The masses continue to reorganize without much change in appearance. Three or 4 days after centrifuging small rounded elevations appear which later become hypostomes, and near these are one or more small knoblike tentacles. These tentacles, however, are not always associated with the hypostome elevations but may appear at other places on the aggregate (see Figs. 6 and 19). Five to 6 days after centrifuging the hypostomes and tentacles are well developed, and about this time the mass may split into several smaller masses, or rather groups of hydranths and tentacles (Figs. 8, 10, 15, 16, and 17). A detailed description of the development of two or three of these masses from the beginning until the conclusion of the individual experiment will serve to illustrate the development of them all.

The mass shown in Figure 3 was composed of approximately seventy-six pieces of tissue taken from the distal half of the body of two animals, the hypostome and tentacles having been removed, and the proximal half of the body of six animals exclusive of the stalk. The mass represents approximately two-thirds of the total number of pieces of tissue cut; the remainder separated in removal from the centrifuge tube. Twenty-four hours later (Fig. 4) the areas had disappeared and the mass had become homogeneous. Forty-eight to 55 hours later tentacles began to appear (Fig. 5). The mass meantime

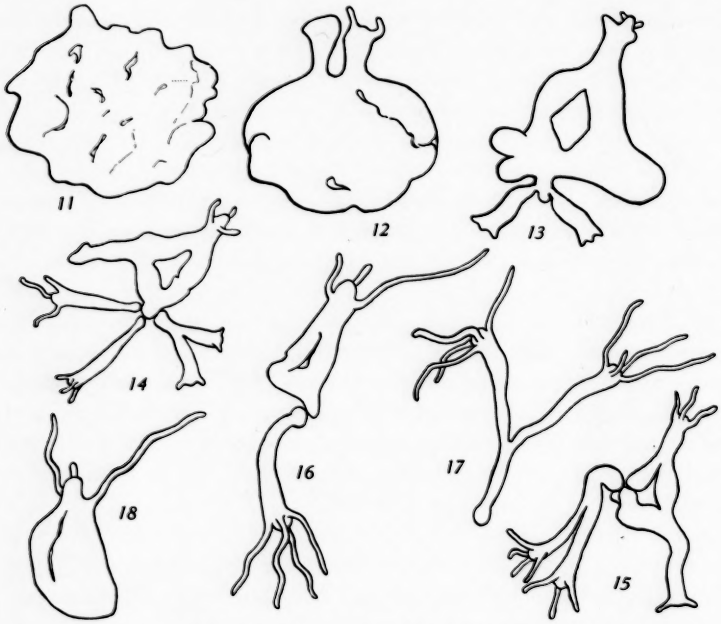
had changed into several cylindrical masses of tissue connected by intercommunicating tubes of smaller diameter. Later two definite



FIGS. 3–10.—Development of a typical aggregate. Figure 3 shows the general form of aggregate or mass with the outlines of some of the component pieces; Figures 4–10, the further development and separation of minor axes. Note that in Figure 10 the original or primary apical region remains in control of the parent-mass.

regions of hypostome and tentacle development appeared (Fig. 6), which 48 hours later became well defined (Fig. 7). Twenty-four hours later the small budlike outgrowth with the two tentacles had been resorbed and a new apical region appeared with four knoblike

tentacles (Fig. 8). Later these two apical regions or polarities formed well-defined normal buds (Fig. 9) and separated from the rest of the mass, which was now apparently under the control of the apical region which first developed. It is found to be true in almost every



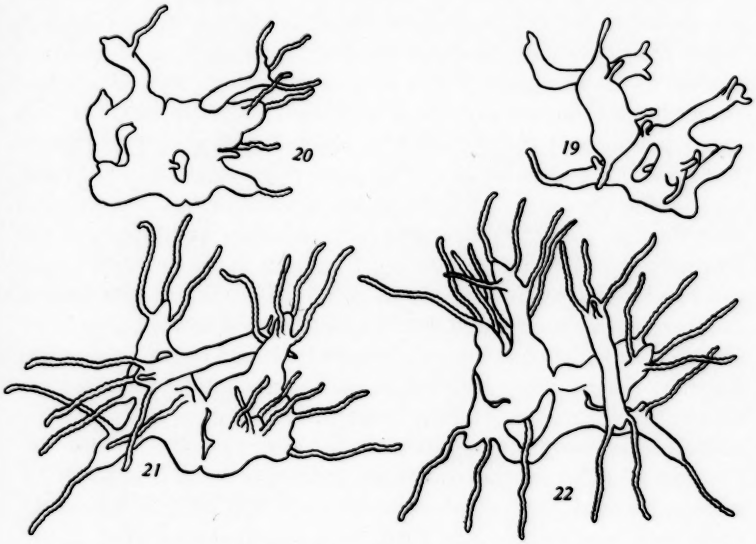
FIGS. 11–18.—Development of aggregate. Note that the primary apical region remains in control of the remainder of the mass. Figure 17 shows a secondary axis with another subordinate axis which has separated from the parent-mass.

instance that the first apical region to develop on the mass assumes control of the mass, and the apical regions which develop later—that is, hypostomes and tentacles—develop into apparently normal buds. These may leave the original mass or remain as more or less well-developed polarities on the main mass (Fig. 10).

Figures 11–18 show the development of another mass or aggregate of approximately one hundred and twenty pieces taken from the body regions of five animals. The two buds first shown in Figure 13

separated from the parent-mass. The biapical or bipolar bud continued to develop and in addition a new apical region (Fig. 15) formed. Both the bipolar (Fig. 17) and the unipolar or normal bud (Fig. 16) eventually left the original mass, which is shown in Figure 18.

Attention was previously called to the fact that it is very difficult to get a fairly compact flattened mass from the pieces which will



FIGS. 19–22.—Inhibiting effect of the differential of contact on development. Figures 19 and 21 show the free or exposed surface and Figures 20 and 22 the bottom or contact surface.

have a well-defined upper and lower surface. In these experiments it was possible to get but four such masses, two of which are shown in Figures 19–30. These are typical and serve to illustrate in general the conditions found in all four. The masses were left undisturbed with the surface relations unchanged except when observations were taken and drawings made. They were then in a few minutes restored to their original position. Figure 19 shows, 48 hours after centrifuging, the free or upper and Figure 20 shows the lower or contact surface of a mass of approximately seventy-three pieces taken from the

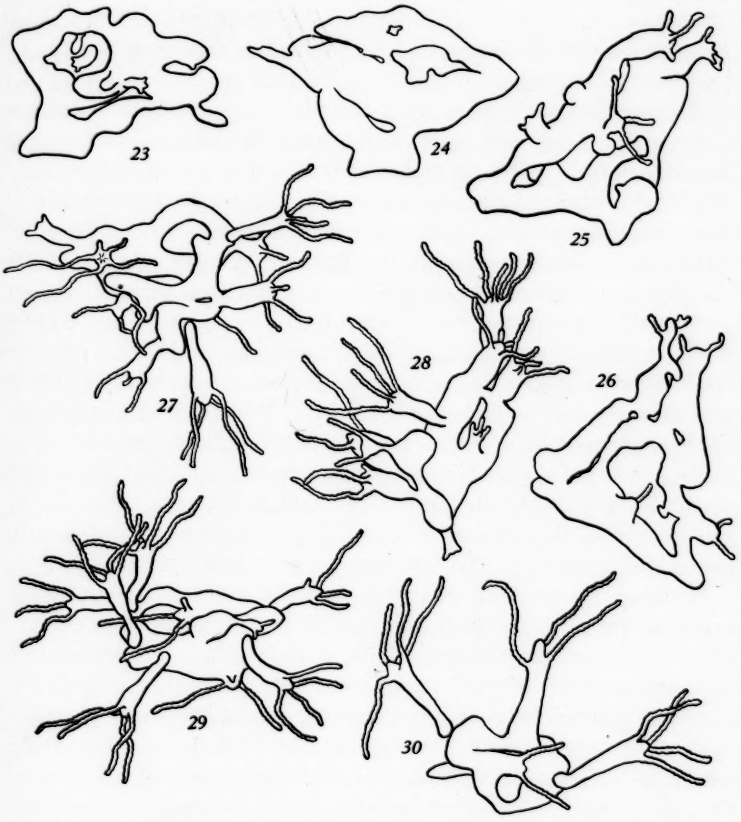
distal halves of the body regions of nine animals. Figure 21 shows the exposed and Figure 22 the contact surface 3 days after centrifuging. If the development of these two surfaces is compared, it will be seen that there are two apical regions developing on the free surface and none directly on the contact surface. Unfortunately, the mass underwent depression and died.

Figures 23–30 show the development of an aggregate made up of one hundred and ten pieces from the distal halves of the bodies of seven hydras. Figure 23 shows the free and Figure 24 the contact surface of the aggregate 3 days after centrifuging. The mass is less compact than the one previously described but there seems to be a more pronounced development of apical structures, particularly tentacles, on the free surface. This same difference is seen in Figure 25 showing the free and Figure 26 the contact surface 5 days after centrifuging. Figure 27 shows the contact surface and Figure 28 the free surface 4 days after sectioning. Figure 27 shows the mass 6 days after sectioning with many of the polarities developed into buds a number of which are about ready to separate. Figures 29–30 show the mass 24 and 36 hours later after the buds had begun to separate from the parent-mass. Unfortunately, the mass underwent depression but recovered and in its subsequent breaking-up into new individuals by budding, apparently, so far as it was possible to observe, the original main axis persisted and seemed to be in control of the mass of material. This point was much more difficult to determine in these large aggregates with their numerous polarities than in the smaller masses since in these latter there were fewer apical regions developed.

DISCUSSION

Studies on the developement of dissociated cells have been made by various investigators. Studies of this type were made on sponges by H. V. Wilson (1907, 1910*a*, 1910*b*); Huxley (1921), Müller (1911), and Galtsoff (1925). Wilson (1911), Child (1928), and Papenfuss (1933) have made studies on dissociated cells of *Coelenterata*, and Issajew (1926) reported on the behavior of dissociated fragments of *Hydra*. Both Child (1928) on *Corymorpha* and Issajew (1926) on *Pelmatohydra* interpret their results in the light of the gradient theory. The report of Papenfuss (1933) has appeared as yet in the form of an

abstract only and no attempt is made in the abstract to correlate the results with the axial gradient theory. Child concludes as a result of



FIGS. 23–30.—Inhibiting effect of the differential of contact on development. Figures 23, 25, and 28 show the free or exposed surfaces and Figures 24, 26, and 27 the bottom or contact surface. Figures 29–30 show the early stages in the separation of secondary axes.

his study on *Corymorpha* that the "polarity of the aggregates is determined by the environmental difference between the upper free surface and the surface in contact with the substratum, and that

polarity is primarily a quantitative differential in physiological condition." Issajew (1926), from his work on dissociated fragments of *Pelmatohydra*, as has been previously pointed out, concludes that the loci of new axes—that is, apical regions—were apparently determined by portions of the peristomes of the original animals as shown by the presence of the original tentacles.

The experiments here reported differ from those of Child (1928) on *Corymorpha* in that aggregates or masses from dissociated fragments are studied rather than aggregates of dissociated cells, and from Issajew (1926) inasmuch as the peristome and tentacles were discarded. The pieces of tissue used were taken from the body region only after removal of peristome and tentacles. Other differences in technique of massing have been previously pointed out. In these other studies as well as the present one, one of the objectives was to determine, if possible, the origin of new gradients or axes. It would seem that by the method of cutting the pieces the original polarity and the relation of dominance and control of the individual animals, whether dissociated into cells or small fragments of cells, would be destroyed. Then in the process of aggregation new environmental differentials are set up not only with the inorganic surroundings but also with the organic—that is, the adjacent cells or cell groups—which would result in changed physiological conditions and relationships.

However, it seems safe to assume that the original rate of physiological activity persists in these small pieces at least to some extent. If, as Child and others have many times shown, the establishment of new axes is brought about by a region of apparently high metabolism establishing dominance over a region or regions of lower rate, then the explanation seems plausible that pieces from those body regions with a high rate of metabolism will become the organizers of new polarities, or the high ends of new gradients which are dominant over regions of lower rate. That such organizing regions are not restricted to the cells of the peristome is clearly demonstrated in these experiments. These data apparently indicate that such organizers would be groups of cells from the more distal body levels and, in budding animals, the budding zones. That such levels do have a higher rate of physiological activity has been indicated by Child and Hyman (1919). These higher levels would therefore develop as apical regions

with characteristic apical structures and determine the development of other parts. Further evidence for this interpretation is found in the reports of Browne (1909), Koelitz (1910), King (1903), Rand (1899*a*, *b*, 1911), and Child (1932).

A polarity so determined, is, in a certain sense, a new polarity but dependent on the persistence from pre-existing individuals of physiological differences in different cell groups. Child (1928) further points out that polarities, if so determined, should result in a high frequency of multipolar forms in these aggregates since these would undoubtedly contain more than one cell group which would become the organizer of a new apical region. As a matter of fact, this is what apparently occurs, for all the aggregates were either bipolar or multipolar forms, the latter decidedly predominating in the large masses.

Other evidence in support of such a theory seems to be afforded by observations made in connection with these experiments. To summarize these briefly, the distal regions of certain animals were stained with Nile-blue sulphate, a vital stain, and these pieces aggregated with those from unstained lower body levels. In the development of the masses the majority of the newly developed apical regions—that is, hypostomes and tentacles—were stained blue. However, these experiments are at present far from complete and the data are by no means conclusive.

Such determinations of polarity as suggested above are independent of the differences brought about by the environment. Child (1926, 1927*a*, 1927*b*, 1928) has found that the factor of differential exposure in relation to substrate is effective in determining polarity in stem pieces of *Corymorpha*. That the factor of differential exposure does play a part in the development of these masses or aggregates seems to be very definitely indicated. However, there are certain difficulties encountered in making direct observations. In the first place, the aggregates do not at any time adhere to the substrate, and, second, soon after centrifuging the mass is capable of contraction and a certain amount of movement, which results in some change of position. This renders impossible the supposition that the lower surface is in close contact with the substrate and that the piece remains continuously in one position. Nevertheless, observations made on both the

free or the exposed as compared to the contact or lower surface show that in all instances there are more developing apical structures—that is, new polarities—on the free than on the contact surface (see Figs. 21–22 and 27–28). It would appear, therefore, that while the factor of differential exposure did not completely obliterate the original physiological condition of the pieces, it apparently had a definite inhibiting influence so far as the region in contact is concerned. In Child's studies (1928) on *Corymorpha* the cell aggregates which had a low death-rate were 0.3–0.5 mm. in diameter. Those above this size soon died. These hydra aggregates were 1–2 mm. in surface diameter with occasional open spaces where there had been fusion. They made a comparatively thin layer—approximately the thickness of the diameter of a normal hydra. To secure masses comparable to those of *Corymorpha* would require cellular dissociation since there is no other way to secure small masses of cells in which the gradient relations would be obliterated by dissociation. Such a small amount of material should reduce the possibility of numerous polarities or multipolar and bipolar forms as in *Corymorpha*.

Evidence from these experiments indicates that the first well-developed apical region to appear on the mass seems to determine a primary gradient in the aggregate. Other apical regions develop as secondary or subordinate axes which develop foot and stalk regions and separate from the parent-mass as normal buds. Sometimes a secondary axis or polarity may consist of a secondary dominant region with a subordinate or tertiary axis (see Figs. 15–17). In case of very large masses another gradient may be set up which may closely approximate the primary or initial gradient. This is particularly true if there is a simultaneous development of two apical regions. In such instances the very large mass was seen to break up into two smaller masses. Other observations which are in agreement with these on the establishment and influence of a primary gradient in multipolar forms were reported by Weimer (1932). Multipolar forms occurred with a primary gradient which was represented by a well-developed apical region and two subordinate gradients each represented by single tentacles. These pieces were fed over a period of time and growth took place. With the lengthening of the piece the two subordinate axes developed into perfect hydranths with body, foot, and

stalk and separated from the parent-mass in the same manner as happens in these large aggregates.

One of the interesting questions growing out of these experiments is the origin of the ectoderm and entoderm. The superficial appearance of the massess seems to indicate that the outer surface is covered with a layer of ectoderm within 2 or 3 hours after aggregation. This apparent early organization is the more interesting in that these masses are made up of dissociated pieces of the body of the animal rather than dissociated cells. In the case of dissociated cells such reorganization might be explained by cell migration. Child (1928) offers the following suggestions for the organization of these layers of the aggregates in *Corymorpha:* first, entoderm cells migrating to the interior and ectoderm cells to the exterior; second, ectoderm cells on the exterior and entoderm cells in the interior may grow at the expense of cells which are out of their usual environment; third, ectoderm or entoderm cells may undergo dedifferentiation and redifferentiation. It would seem to be more plausible in these experiments, considering the apparent early cellular arrangement, to eliminate the latter two suggestions and to hold to the first suggestion of migration.

The assumption that the component pieces were so arranged in the aggregate that original ectoderm was on the exterior and orginal entoderm on the interior is apparently untenable. It would seem from the way these pieces were jumbled in the tube without thought of arrangement and the added change in relation due to centrifuging that there would be certain pieces with the cell relations reversed. Full discussion of this point is reserved for a subsequent paper when a histological study will be reported of sections prepared from masses at varying periods of development.

SUMMARY

1. Small pieces of the body region of *Pelmatohydra oligactis*, after removal of hydranth and stalk, will aggregate into masses when centrifuged at a rate of approximately 750 r.p.m. for 1 hour. Such masses have been made up of as many as one hundred and twenty pieces from the body regions of five animals. These masses usually develop into multipolar forms.

2. These masses appear to have definite ectodermal and endodermal cell arrangement 2–3 hours after centrifuging.

3. Thirty-six to 48 hours after centrifuging tentacles and hypostomes begin to appear on these masses. Seventy to 90 hours after centrifuging apical regions are well developed.

4. The first well-developed apical region with hypostome and tentacles seems to be the high end of the primary gradient or axis of the mass and to exercise control. Other apical regions or secondary axes develop which separate as buds usually from the parent-mass.

5. The determination or organization of these new polarities or axes seems to be brought about by pieces or cell groups from those body regions with a high rate of metabolism which are incorporated in the mass and which retain a high rate of physiological activity.

6. The relatively small number of apical regions developing on the contact or bottom surfaces of these masses would seem to indicate that the factor of differential exposure, while not completely obliterating the original gradient conditions in the pieces, does have a well-defined inhibitory effect on development.

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THE DIFFERENTIATION OF HEPATIC AND PANCREATIC TISSUES OF THE CHICK EMBRYO IN CHORIO-ALLANTOIC GRAFTS[1]

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THE differentiation of the early hepatic rudiment into apparently normal liver tissue when grafted to the chorio-allantoic membrane has been reported by several workers. Hoadley (1925, p. 150) makes note of it in his grafts of heart level of 37-hour chick embryos, Hunt (1929) found it in grafts of node level taken in the advanced head-process stage, and a similar finding was reported by Willier and Rawles (1931*a*) in their study of grafts of whole blastoderms in early stages of development. In general, these observations show that the hepatic tissue which differentiates from grafts of parts of the early blastoderm is histologically normal.

On the other hand, Murphy (1916) and Willier (1924) found that adult liver tissue when grafted to the chorio-allantoic membrane ceased to grow and underwent extensive degeneration. The former found in his grafts of adult chicken liver that there was a lack of growth and an intense infiltration of the tissue by leucocytes. Willier alluded to this same phenomenon in his paper on the effects of thyroid grafts on the chick embryo, pointing out that grafts of liver tissue from newly hatched and adult chickens will not grow when grafted to the chorio-allantoic membrane. Instead, apparently the only parts that persist are the bile ducts.

With these results in mind, experiments on hepatic tissue were made to determine the time at which the liver, having ceased to grow and differentiate, becomes degenerated, and if possible the underlying causes for this change.

The work on the liver came naturally to include the pancreatic tissue, and a comparable study of that gland was undertaken also, to ascertain whether at a definite period it too would show a similar

[1] I wish to express my appreciation to Dr. B. H. Willier for the many valuable suggestions and criticisms which he has so generously given throughout the work.

cessation of growth and a subsequent degeneration, in chorio-allantoic grafts. In 1924 Danchakoff reported normal differentiation of pancreatic tissue transplanted from donors of 7 days' incubation, and other workers (Willier and Rawles, 1931*a;* Hunt, 1929; Hoadley, 1925), using pre-rudimentary stages, also found pancreatic tissue which was apparently normal in its differentiation. Reports other than these on the behavior of early embryonic material have not been found in the literature; consequently, the experiments on pancreatic tissue recorded here form a more extended group than do those performed in connection with the work on the liver.

MATERIAL AND METHODS

Embryos of Single-Comb White Leghorn chickens were used for both the hosts and the donors. The type of isolation used in the experiments varied in accordance with the age of the donor. In the very early stages of development (i.e., up to 3 days) complete isolation of the rudiments appeared impossible, so that three different regions of approximately the same level were grafted independently: (*a*) a piece of the floor of the gut just large enough to include the rudiment of the liver, (*b*) a piece of the roof of the gut large enough to include the diverticulum of the dorsal pancreas, and (*c*) a ring of gut tissue of about the same size including both of these rudiments. The isolated pieces were not measured, but were for the most part less than 1.0 mm. square. In the case of older donors, the pieces either consisted of the primordium alone or included also a small part of the adjoining region of the gut. The purpose of this last isolation was to test the influence of the gut tissue on the development of the gland rudiment. In the series of pancreatic isolations, donors of 10 or more days' incubation were handled in two ways. In most cases the gland was implanted in fragments because of its size, but in a few instances the entire gland was grafted for purposes of comparison. The instruments used to make these isolations were fine ear knives, one with a single cutting edge and the other with a double spearlike edge.

In the experiments on the liver, some of the donors were incubated for 32–35 hours (18–20 somites) and others for 3, 4, 5, and 6 days. The implants for the pancreatic series were taken from donors in-

cubated from 3 to 19 days. The original method of grafting, described by Willier (1924, p. 68), was used, with the modifications already noted. The implants were placed on hosts of 8 or 9 days' incubation and were allowed to remain until the host was 18 days old, when they were removed and prepared for histological examination.

Both Bouin's solution and Zenker-formal were used as fixatives, and the sections were cut at 6 or 8 micra from paraffin blocks. The hepatic grafts were stained in Heidenhain's iron hematoxylin and counterstained with Orange G. This combination was also used as the routine stain for the pancreas sections. From several of the grafts, however, test sections were set aside and stained with special dyes to determine whether an apparently normal pancreas was physiologically active. For these tests a few sections from each slide of the serial were mounted separately; the object was to obtain as representative an area as possible for the reaction of the dye. Stains specific for zymogen granules, which occur in the acinar tissue, and for the alpha and beta cells of the island tissue were employed. Neutral gentian demonstrates the zymogen granules well but the stain fades very quickly, consequently Mallory's azan stain and Bensley's combination of aniline acid fuchsin and methyl green were the stains mainly used since they are specific for both types of tissues. By the former method the alpha and beta cells are differentiated with red and yellow granules, respectively, and the zymogen granules are red; whereas with the latter, the zymogen granules and the granules of the alpha cells are red and the beta granules are blue. The azan stain is a particularly good one, for it has little tendency to fade.

For purposes of comparison, the normal development of these glands was followed; through the fifteenth day of development for the liver, and in the case of the pancreas up to the time of hatching. The staining methods used in the experimental work were also employed in these series of normal development.

STAGES IN THE DEVELOPMENT OF THE HEPATIC AND PANCREATIC RUDIMENTS AT THE TIME OF IMPLANTING

The liver.—The hepatic rudiments used for transplantation were taken from donor embryos incubated 3, 4, 5, and 6 days, thus covering the early developmental changes in the gland.

The liver first appears as a ventral outpushing of the gut at the 22-somite stage, just as the right and left wings of the splanchnopleure come together in the median line. In the 35-somite chick, as the gut continues to close a second ventral outpushing appears, a little posterior to the first. At 68 hours the posterior diverticulum develops a bud which represents the primordium of the gall bladder. At first the two anlagen are distinct, and each gives off branches which unite into a close network of hepatic cords.

At the end of the third day of development, the hepatic cords of the two rudiments have anastomosed with each other to form a single organ, which is subsequently bilobed. As the two liver buds come together, they surround the vitelline veins, and by the fourth day, incipient blood capillaries can be seen in the mesenchyme between the hepatic cords. At this stage the gall bladder is a small but definite pouch, although it does not begin to store bile until the sixth day of incubation. During the fifth and sixth days, the solid columns of hepatic cells continue to grow and anastomose until the organ consists of a compact mass of characteristic polyhedral cells with large nuclei and occasional vacuoles in the cytoplasm. The solid cords acquire narrow lumina which are the bile capillaries. The blood capillaries become sinusoidal and connect with the vitelline veins.

The pancreas.—The pancreatic tissues used in the transplantations were taken from embryos varying in age from 3 to 19 days of incubation. This was found necessary since in this gland the changes occur more gradually than they do in the liver. In the very earliest operations only the dorsal diverticulum was grafted, whereas after the ventral diverticula appear all three were transplanted.

According to Lillie (1919), the pancreas develops from one dorsal and two ventral diverticula. The dorsal one arises first from the roof of the gut at the level of the anterior intestinal portal in the 35-somite chick. Following this, on the fourth day (96 hours) the right and left ventral outpushings arise from the common hepatic diverticulum near its union with the duodenum. Clara (1924), in a report on the pancreas of the bird, gives a summary of the constitution of the gland and his statements agree with those of Lillie. Among the birds that Clara included were the chick, the sparrow, the pigeon, and the duck; but since his interest was largely histological, he did

not investigate the embryological foundations of the gland. He refers to three lobes, two principal ones and one very small "spleen segment," which he thinks correspond to the three original anlagen.

During the fourth and fifth days of development, the primary outpushings grow, become branched, and extend into the surrounding mesenchyme, where they form distinct glandular masses. At this time a complete isolation of the ventral rudiments is impossible because of their close association to the common bile duct. By the close of the sixth and on the seventh day of incubation the dorsal diverticulum has extended into the mesenchyme surrounding the ventral rudiments, forming the foundations of the adult organ. At this stage, with care it can be isolated from the liver. In an embryo of 7 days' incubation the gland consists of a few tubules imbedded in mesenchyme; the tubules are connected to the gut by one or more ducts.

By the tenth day the pancreas has become a compound tubular gland with many branched tubules terminating in small acini. Activity of these acini is demonstrated by zymogen granules which, with Mallory's azan stain, stand out as sizable red particles. At 12 days the tubules are more branched and the stroma is so condensed that the gland has a more compact appearance. The Islands of Langerhans make their appearance at this time, first as isolated alpha and beta cells scattered between the tubules and acini. The island tissue is easily recognizable in sections treated with azan stain. The many beta cells contain small yellow granules whereas the comparatively few alpha cells exhibit large red granules. By the fifteenth day the islands have increased in number and size so that the whole gland assumes the appearance of the adult organ.

THE GRAFTS

A total of 198 grafts has been examined histologically: these include both liver and pancreatic tissues in seven types of isolations.

In general, the blood supply of the grafts is somewhat variable. The theory was that this condition might bring about histological changes in the tissues of the grafts, but actually it was found that such changes depended on the age of the tissue rather than upon the richness of the blood supply from the host.

1. GRAFTS FROM A PIECE OF THE FLOOR OF THE GUT LARGE ENOUGH TO INCLUDE THE LIVER RUDIMENT

Donors with from 15 to 28 somites furnished the isolations for this series of experiments. The part to be grafted, approximately 1.0 mm. square, was taken from the floor of the gut at the level of the anterior intestinal portal, and an effort was made to avoid including the side walls of the gut. Nineteen grafts were sectioned and in 9 of them small areas of liver, gut, and pancreas were observed. In addition, 2 of the 9 cases contained bits of heart tissue. Of these 9 grafts 2 were from donors of 15 somites and the remaining 7 were from donors of 16, 18, 21, 23, 25, 26, and 27 somites. In an abstract reporting the early part of this work (Holton, 1929), it was stated that neither gall-bladder nor pancreatic tissue occurred in the grafts. Although the sections were not stained selectively for pancreas in the early work, further study has shown that the tissue was present and an epithelial vesicle identified as gall bladder occurred in numerous cases.

Hepatic tissue is identified as a compact mass of tubules and sinusoids, connected either with the gut tissue of the implant or with the gall bladder when that structure is present. The sinusoids which are united with the host blood vessels contain blood corpuscles. The tubules consist of cells with large, rounded, darkly staining nuclei and vacuolated cytoplasm, thus conforming cytologically to normal liver tissue of a corresponding age. The small lumina surrounded by these cells correspond to the bile capillaries of the normal tissue. In the resting cells the cytoplasm and nuclei are normal, but many of the cells appear to be in stages of mitosis, indicating growth in the graft.

The pancreas appears in only 2 of the 9 grafts (23 and 28 somites), but in these instances it is identified as an irregular mass of tubules connected by ducts to the gut tube. The tubules may be acinous, but with all the stains tried they appear to be simply lumina with cuboidal cells surrounding them, for the characteristic zymogen granules, which normally appear on the tenth day, are entirely lacking. The absence of the zymogen indicates that the tissue is not functionally normal 9 days after its transplantation to the host. Between the tubules a great deal of island tissue is present. This tissue

is identified at first by the peculiar bluish granules of the beta cells following the iron-hematoxylin stain. In the azan stain the beta cells are recognized by their yellow granules and the alpha cells by their red granules. These observations seem to place the development of the islands in the graft slightly in advance of those in a normal pancreas, for definite islands are usually not found before the fifteenth day.

2. GRAFTS FROM A PIECE OF THE ROOF OF THE GUT LARGE ENOUGH TO INCLUDE THE DORSAL PANCREATIC RUDIMENT

The 19 grafts of this series were obtained from donors of 64–90 hours (35–44 somites) and consisted of small pieces of the roof of the gut at the level of the anterior intestinal portal, and large enough to include the dorsal pancreatic diverticulum. Some were taken just before, and others just after, the appearance of the primordium. All showed development of pancreatic tissue except one (68-hour donor with 35 somites). Of these transplants, 6 were grafts from donors of 89–90 hours, after the appearance of the primordium, 6 were from donors of about 75 hours, and 7 came from donors of 64–68 hours, before the actual appearance of the outpushing. In 4 of the 6 grafts in which the pancreas was isolated shortly after its appearance, small amounts of hepatic tissue were identified. As in the foregoing series, the pancreatic tissue consisted of normal islands and tubules connected with the gut by ducts.

3. GRAFTS OF A RING OF THE GUT TUBE INCLUDING ALL OF THE LIVER AND PANCREATIC RUDIMENTS

These grafts were taken from donor embryos of 15–38 somites and consisted of cross-sections of the gut at the level of the existing anterior intestinal portal, not more than 1.0–1.5 mm. in width. Of the 24 grafts which were sectioned, 18 were from donors of 35–38 somites and the remaining 6 came from donors of 15, 16, 19, 21, 26, and 29 somite stages. All but 1 of these (21 somites) developed hepatic tissue which was apparently normal, i.e., it corresponded closely to the liver from a normal 11-day old embryo and conformed to the description of the grafted liver tissue given above.

Tissue typically pancreatic in its arrangement has been observed in these grafts, but because the material was fixed in Bouin's solu-

tion, its characteristic components could not be identified by selective staining methods.

In all of these transplants there appear to be a few free granulocytes, especially between the tubules of the liver. They are not localized but are scattered at random, more occurring in some areas than in others.

In addition to the foregoing 24 grafts of liver and gut tissue, 4 grafts of the liver rudiment free from the adjacent gut from donors of 3 days' incubation are included here. In these the hepatic tissue which is formed is identical with that found in grafts of both rudiment and adjoining gut. It is clear that the most normal hepatic tissue occurs in grafts obtained from donors during the third day of development.

4. GRAFTS OF LIVER RUDIMENTS AND TISSUES FROM OLDER EMBRYOS

A total of 41 grafts has been examined histologically and a summary of the results obtained is presented in Table I. Twenty-five of these grafts were obtained from transplants in which hepatic tissue alone was isolated, and in 16 grafts a piece of adjacent gut was included with the liver tissue. The purpose of the two types of isolation was to determine if possible whether the tissue had the same capacity for differentiation in the presence or absence of the gut. In the older embryos, owing to the large size the of liver, pieces were transplanted instead of the whole gland. In several grafts of such cases histological examination showed a lack of gall-bladder epithelium.

Twenty-three grafts were examined from donors of 4 days' incubation—13 of them contained hepatic rudiment alone and 10 included liver and gut. The hepatic tissue which differentiates in these grafts is essentially normal. The cells of the hepatic cords, however, are more vacuolated, and mitotic figures are less frequently seen than in the grafts of the younger stages. The sinusoids are apparently more numerous. The fact that mitotic figures do occur, and that the graft is large, shows that growth has not completely ceased. In these transplants there is a slight increase in the number of granulocytes over the number found in grafts of younger stages and in the normal

liver. They occur in masses among the hepatic cords in some areas, others being entirely free of them.

Seven grafts were studied from embryos of 5 days' incubation. Of these, 6 were a combination of liver-and-gut tissue, and 1 was a portion of hepatic tissue alone. All the grafts give evidences of degeneration in the hepatic tissue. The granulocytes, which are now found

TABLE I

SUMMARY OF RESULTS ON LIVER DIFFERENTIATION

Donor Age (Days)	Total Age of Grafts (Days)	No. Grafts Examined	Histology of the Graft			
			Liver Tubules	Sinusoids	Infiltration	Necrosis
15–28 somites..	11	19	Normal; mitoses present	Numerous; connected with host blood vessels	Few	None
3.....	12	4	Normal; mitoses present	Numerous; connected with host blood vessels	Few	None
4.....	13	23	Some cells vacuolated; few mitoses	Enlarged	Definitely localized	None
5.....	14	7	Some normal; some abnormal	Enlarged	Throughout grafted tissue	Traces
6.....	15	11	Nuclei and cell membranes degenerated	Empty	Heavy throughout graft	In extended areas

spread indiscriminately through this tissue, have apparently engulfed the hepatic cells, taking their places in some of the tubules. The result is a transformation of the tubules, ranging from normal ones with clear-cut central lumina and typical cells to abnormal ones with lumina surrounded by cells in all stages of degeneration. The process may even go so far as to leave only a reticulum around the lumen of the hepatic tubule, the cytoplasm and nuclei being entirely gone.

From 6-day donor embryos, 11 grafts of hepatic tissue were examined histologically. The implants are all of considerable size, in-

dicating good growth; but actually the size is apparently due to the presence of large watery vesicles or to gut tissue, the amount of liver tissue being less than the amount originally transplanted. Moreover, the liver tissue is difficult to recognize in most cases, since the hepatic cells are highly degenerate, i.e., the cells, nuclei, and cell membranes have disintegrated, leaving only traces of their outlines in the mesenchyme. The sinusoids have increased in width and contain very few blood corpuscles. The infiltration of granulocytes is very heavy throughout the mass of hepatic cords. On the whole, the picture is one of extensive degeneration. These experiments show that embryonic liver tissue, grafted to the chorio-allantoic membrane, first shows a cessation of growth and differentiation on the eleventh day of its development, and by the fourteenth day its degeneration is complete. From the work of Murphy and others it is known that adult liver will not persist in chorio-allantoic grafts, consequently no further experiments on later stages were attempted.

When gut is included with the liver rudiment or tissue in the graft, the granulocytic invasion is slightly retarded and the vacuoles in the cytoplasm of the hepatic cells are not as numerous as in the cases where gut is absent. Furthermore, it should be noted that in the majority of cases where gut is included in the isolation, gall-bladder epithelium and pancreatic tissue occur and both seem to be in a normal and somewhat active condition.

5. GRAFTS OF PANCREATIC TISSUE FROM DONORS OF 4–19 DAYS' INCUBATION

Since the pancreas is made up of three distinct parts—acini, ducts, and islands—and each part develops at a different time in the embryonic life, it was necessary to use donors which varied in age from 4 to 19 days of development. The pancreas isolated included the ventral primordia up to 7 days of development, at which time the dorsal diverticulum has extended into the same mesenchyme and consequently is also included. A brief summary of the results of these operations will be found in Table II.

The first experiments deal with donors of 4 days' incubation. Before this time, on account of the close connection of the liver, a complete isolation is practically impossible. Almost as common as the

appearance of hepatic tissue in these grafts is the presence of sympathetic ganglia. These are always found in with the tubular tissue of the pancreas and doubtless were included with the original isolation. The earlier attempts to make pure isolations were not always

TABLE II

SUMMARY OF RESULTS ON PANCREAS DIFFERENTIATION

Donor Age (Days)	Total Age of Grafts (Days)	No. Grafts Examined	Histology of Graft					
			Pancreas			Granulocytes	Necrosis	Invasion of Connective Tissue
			Islands	Acini	Ducts			
64–90 hours.	11–12	19	More than normal	Normal but no zymogen granules	Normal	Few	None	None
4–6....	13–15	13	Still more abundant	Normal but no zymogen granules	Normal	Few	None	None
7–8....	16–17	9	Greatest quantity	Normal but no zymogen granules	Normal	Few	None	None
8–9½...	17–18½	13	Slight decrease	Normal but no zymogen granules	Normal	Few	None	Noticeable
10–11...	19–20	11	Still greater decrease	Normal but no zymogen granules	Surrounding connective tissue increased	Slight increase	Trace	More abundant
12–13...	21–22	9	Still greater decrease	Normal but no zymogen granules	Surrounding connective tissue increased	More abundant	Trace	Still more abundant
14.....	23	7	Very little	Acini reduced	Surrounding connective tissue increased	More abundant	Trace	Still more abundant
15–16...	24–25	10	None	Acini reduced	Very small	More abundant	More extensive	Very extensive
17–19...	26–28	19	None	Very compressed	None present	Maximum number	Maximum amount	Maximum amount

successful since in several grafts gall-bladder epithelium was found and in one case heart tissue was identified.

Comparatively few transplants were studied in which the gut was included with the pancreas, because the presence of the former seemed to make no appreciable difference in the differentiation of the latter. In all grafts the pancreatic tissue is in acinar form, but every section stained with specific stains shows the cells to be devoid of the characteristic zymogen granules, the indicators of secretory

activity. Furthermore, the result is the same regardless of whether a piece (either a half or a third) or the whole of the embryonic pancreas from donors of 10 days or older is grown in a graft. In each case the connective tissue is found invading the pancreatic tissue, necrotic areas are present, and masses of granulocytes are found. On the basis of general experience in chorio-allantoic grafting, however, it seems logical to assume that the smaller piece gives the more typical picture.

In grafts obtained from donors of 4–8 days, the island tissue is so extensive that a section stained by the routine method displays the beta cells with unmistakable clearness, and in the best sections they seem much more abundant than in the normal pancreas. In the majority of these grafts the number of free granulocytes is not appreciably greater than the number in the normal chick pancreas of a corresponding age, where they are more abundant than in at least some of the organs and tissues examined. It is interesting to note that where liver and pancreas occur in the same graft, the granulocytes are more abundant in the former.

Certain changes are seen in the grafts of pancreatic tissue of 8- and 9-day donors. For instance, in several there is a slight but definite decrease in the occurrence of island tissue, and occasionally an increase in the amount of connective tissue between the tubules. This slight increase is apparently significant when considered in the light of observations made on older stages.

In the transplants obtained from donors of 10–14 days, a very noticeable decrease is noted in the amount of island tissue. In grafts of pancreatic tissue from 14-day chicks the islets are entirely lacking in 5 of the 7 cases examined. In the other 2, a few alpha and beta cells in the midst of dense aggregations of granulocytes represent the former endocrine part of the tissue. In some instances the granulocytes are so abundant as to obscure even these few cells. With the decrease of the island tissue there is a simultaneous increase in the number of granulocytes. Also the amount of connective tissue present is much greater in a graft of a 14-day chick than in one from a 10-day donor, showing that this process, too, is a progressive one.

The grafts of pancreatic tissue from donors of 15–19 days are most striking in that no island tissue is to be found in any of them.

The tissue is lobulated, but only because large amounts of connective tissue have developed between the tubules, so that they are greatly compressed and reduced to a size much smaller than normal. The granulocytes are present in great masses, typically occurring around and between the compressed acinar-like tissue.

GENERAL DISCUSSION

These experiments show that the hepatic rudiment of the chick embryo has the capacity for independent differentiation into specific hepatic tissue. Hepatic rudiments from donors which have been incubated up to 3 days differentiate normally into cells of a characteristic nature, these cells forming tubules that are separated by many typical vascular sinusoids. The outstanding difference between the hepatic tissue of the graft, and that of a control embryo of a corresponding age, is its arrangement. In the graft it is always in an irregular mass with no organization into lobes. The form and structure of the hepatic tissue in these early grafts is the same as that described in the paper of Willier and Rawles (1931*a*, p. 90) on the developmental relations of heart and liver. In all grafts in which liver tissue attains less than a total age of 12 days, it is histologically normal. Beginning at about this time, a change is indicated by an increase in the size and number of vacuoles occurring in the hepatic cells. This process is slightly retarded if the piece of adjacent gut is included with the liver rudiment in the isolation. Coincident with this change in the hepatic cells is a noticeable increase in the numbers of free granulocytes appearing in occasional areas of the liver tissue. The grafts from older donors show these changes even more definitely, and, finally, when they have reached a total age of 14 days, the hepatic cells have undergone such an extensive degenerative process, and the granulocytes occur in such great numbers throughout the graft, that it is no longer possible to distinguish the tubules.

The reason for the onset of the degenerative process is not known, but the fact that it occurs regularly at a certain time in the differentiation of the gland in the graft suggests that a specific change of some sort acts at this time, which renders it incapable of further development outside of its natural and original environment. Other

workers in this field have noticed a similar behavior of the liver tissue. In a personal communication Willier states that in chorio-allantoic grafts of liver tissue from hatched chick donors, only the bile ducts remain in the midst of an intense infiltration of leucocytes, the liver cells apparently having undergone disintegration. Also the descriptions by Murphy (1916, p. 2) and Willier (1924, p. 90) of this reaction in the grafted liver tissue indicate that the result is a constant one.

This peculiar degeneration of liver tissue in the graft may be correlated with the biochemistry of the organ. From the work of Hanes (1912), Heaton (1926), Needham (1927), and others, it has been shown rather conclusively that on the eleventh day, and shortly thereafter, there is a physiological change of special significance taking place in the developing chick. In tissue-culture studies of chick liver, Heaton (1926) noticed that the outgrowth of embryonic liver of 11 days' development changed morphologically and physiologically from epithelial cells to cells with reactions like those of fibroblasts. This is natural since up to the present time it has been reported that all cells kept in culture for a time tend to look like fibroblasts, but the significant and unexplained feature is that this change occurs on the eleventh day and not before. Furthermore, Needham (1927) has shown by means of graphs and descriptions based on chemical analyses of whole embryos that there is a distinct change in the energy source from protein to fat, and a marked storage of glycogen commencing on the eleventh day of incubation. Also Hanes (1912) has noted a substitution of the phosphorized fats by cholesterol esters in the liver, on the fourteenth day of development, which is shortly after the physiological change noted by Needham. The findings of all these workers seem to suggest a significant correlation between the degeneration in grafted tissue and certain physiological changes occurring in normal hepatic tissue at approximately the same time.

The loss of capacity to maintain a high state of differentiation on the part of the tissue in the graft has been noted by others. Hiraiwa (1927), working with rat embryos, found that very early in development the tissue from the central nervous system ceased to develop in a chorio-allantoic graft. No mention was made of a species speci-

ficity, but it might possibly have been an explanation in this case. Sandstrom (1930), working with duck-kidney tissue grafted to the chorio-allantoic membrane of a chick, obtained a similar result and attributed it to an effect of species specificity. His later observations, however (1932), in which he grafted duck tissue to duck host, seem to point to a lack of function of the tissue in the graft. E. A. Hunt (1931) has found the same thing true of grafted skeletal muscle. In her case there could be no question of a species specificity because the donors and hosts were both chick material. Likewise in the present work, the degeneration of the grafted liver tissue may be due to a lack of function in the graft. The recent work of Doljanski (1931) on chick-liver tissue cultured in a specially treated artificial medium may in a way lend support to this interpretation of a lack of function. He cultured the liver tissue in a medium which had been so treated that the fibroblasts could not live. In this medium the liver cells grew for months as so-called pure cultures of specific hepatic cells, and Doljanski attributes this to the fact that the liver cells, being functioning entities in themselves, could manufacture that which was lacking in the medium and so continued to exist and grow. Possibly the ability to function was what kept the cells in the culture alive, and conversely in the presence of a host which is functioning there is no stimulation of the grafted tissue which as a result degenerates.

The problem of differentiation of the pancreatic tissue in grafts is a more complicated one since both the exocrine and endocrine components are represented. In grafts taken of the rudiment stages of the gland up to those obtained from donors of 8 days, the tissue appears to differentiate normally. The cells of the tubules are arranged in groups as they are in the secreting acini found in the normal pancreas. However, with the aid of specific stains it is found that the apparently normal groups of acinar-like cells, even from the older donors, completely lack the zymogen granules. A comparison of these results with the normal condition in which zymogen granules are first noted on the tenth day indicates a suppression of the secretory function.

That this apparent suppression of zymogen production is due to the isolation of the gland from an active gut is suggested by the

work of Grauer (1926). He ligated the pancreas of the rabbit and found as a result a period of retrogression at first. After this period secretory activity is apparently renewed, but since no outlet is afforded, absorption of the acini begins and by the end of 19 days they are completely replaced by trabeculae of connective tissue. In chorio-allantoic grafts of pancreas, an effect similar to ligation is obtained, for the gland is growing independently of a functioning gut system and this may serve to suppress the secretory function of the acini. A piece of the gut grafted with the pancreas does not change the reactions of that gland in the least, and this is perhaps to be expected since only a piece of the gut is present and so cannot be functioning as in the normal.

In grafts of the pancreatic rudiment from embryos of 3–9 days' incubation the island tissue is far more abundant than in the normal pancreas of embryos of corresponding ages. For instance, the pancreas rudiment after it has attained a total age of 12 days in the graft is full of island tissue, whereas in the normal embryo of this age the alpha and beta cells are just appearing. In grafts of pancreatic tissue from older stages, however, a gradual but definite decrease in the number of islands present is seen. At first, in grafts obtained from donors of 10 and 11 days, this decrease is slight, but in 5 out of the 7 cases examined from 14-day donor embryos, there is a total lack of alpha and beta cells and none of the grafts from donors of 15–19 days' incubation shows any islet cells whatever.

The acceleration of island production in the graft is similar to an increase of island tissue noted by Grauer (1926) when the ducts of the pancreas of the rabbit were ligated. However, the decrease and disappearance of these cells in the later stages is in marked contrast to his statement that the island tissue remains growing for months after ligation (p. 251). In the rabbit the islands persist apparently for the reasons that (*a*) their secretion is poured into the bloodstream through normal channels and (*b*) they are the only source of secretion in the animal. In the grafts, although the blood supply is intact, the island tissue gradually decreases as the age of the donor increases, so the phenomenon must be traced to some other cause.

The unusual condition, whereby the amount of island material of grafted embryonic pancreatic tissue undergoes an initial increase

followed by a progressive decrease, is as yet unexplained. The acceleration of production which occurs when younger tissue is transplanted may be interpreted as a response to an approaching functional need in the host during the first few days after the graft was made, since in the normal embryo the islets do not begin to appear before the twelfth day and are not well formed until about the fifteenth day. For example, a rudiment of a 4-day donor embryo develops in the graft for a minimum of 3 days before the host island tissue begins to secrete, as is indicated by the appearance of secretion granules in the alpha and beta cells. The gradual decrease and final disappearance of the island tissue in the grafts, noted as the age of the donor increases, is perhaps accounted for by the onset of island activity in the host. This situation should be investigated further by grafting pancreatic rudiments from early donors to hosts in which the endocrine function has already appeared. If the active islands of the host are the influencing factors, then no islands would be expected to appear in the graft. Similarly, further data may be obtained from a study of the pancreatic tissue of older donors, in grafts removed just before the endocrine function of the host begins. The island tissue of such grafts on the basis of this interpretation would be expected to show continued growth and differentiation and probably an increase, as is seen in the grafts from younger donors.

On the other hand, an important factor influencing the observed histological changes may be the age of the pancreatic tissue of the donor at the time of its implantation. Since all of the grafts are on the host for the same length of time and so are equally subjected to its endocrine activity (from the twelfth to the eighteenth day of incubation), it is possible that the age of the grafted tissue rather than the hormone of the host is responsible for the results obtained in the grafts. Further evidence for this interpretation may be obtained from the experiments stated in the preceding paragraph in which the functionally differentiated island tissue is removed from the chorioallantoic membrane before the host islands begin to secrete. If degeneration rather than hypertrophy of the island tissue is found in the grafts, the importance of the age factor would be indicated. This interpretation is in line with the observations made previously, that liver tissue differentiates normally until it reaches a stage in

which it is functionally differentiated, at which time it degenerates—a situation which has also been described for the metanephros (Sandstrom, 1932).

In the present grafting experiments both the liver and the pancreas rudiments are incapable of forming organs of normal shape. In each case the grafted tissue occurs in irregular masses, no lobes or lobules being formed, and often the tissue occurs in a number of groups. This happens whether the rudiment alone or in conjunction with a piece of gut tissue is transplanted, and also whether the whole gland or only a fragment of it is grafted. The failure of the normal form of the organs to appear in the graft introduces an interesting question. Pancreatic grafts obtained from older donors often present a distinct appearance of lobulation, noticeable macroscopically as well as histologically. However, this seems to be due rather to the thickened trabeculae of connective tissue in and around the glandular masses than to a normal differentiation.

Willier (1931*a*, p. 298), in a discussion of this problem with respect to the liver, suggests that "the absence of a septum transversum and of the coelom (the liver in the graft is surrounded by mesenchyme except when and where attached to the heart) are possible factors necessary for the determination of form in the liver." Murray and Huxley (1925), in studying grafts of chick tissue, state that self-differentiation is essentially a chemical process, which leads to the production of specific tissues in definite amounts; but the assumption of particular morphological form must be largely determined by physical (i.e., mechanical) influences. Accordingly, it may be presumed that such influences are lacking in these grafts.

Beginning on the eleventh day of hepatic and about the twentieth day of pancreatic development, there is an infiltration of granulocytes in these tissues, reaching the greatest concentration on the fifteenth day in the former and the twenty-eighth day in the pancreas. This progressive increase in granulocytes parallels the degeneration of the hepatic tissue, or, in the case of the pancreas, the disappearance of island tissue. A similar infiltration of leucocytes has been noted by Murphy (1916, p. 3) and Willier (1924) in grafts of liver tissue, and by Danchakoff (1918) in grafts of the spleen. This process may go so far as to extend to certain organs of the host em-

bryo, e.g., spleen and liver, as described by Willier. On the other hand, Willier (1927 and in a personal communication) reports that commonly such infiltration and degeneration does not occur in grafts of the Wolffian body and other embryonic tissues. Neither Hoadley (1925) nor Hunt (1931) mentions it in any of their experiments on grafting of various embryonic areas. It is thus evident that a definite correlation exists between the invasion of the granulocytes and the process of degeneration and necrosis—that is, whenever degeneration sets in, granulocytes appear.

SUMMARY

1. This problem is an attempt to analyze the growth and differentiation of liver and pancreatic primordia and tissues of chick embryos as tested in chorio-allantoic grafts. The host embryos were usually 9 days old at the time of implanting; the grafts were permitted to grow for either 8 or 9 days.

2. In the earliest grafts the isolations were of three sorts: (*a*) a piece of the floor of the gut large enough to include the hepatic diverticula, (*b*) a piece of the roof of the gut including the dorsal pancreatic rudiment, or (*c*) a ring of gut tissue including the rudiments of both glands. From donors of 3 days or more, the gland was either taken alone or included with a small portion of the adjoining gut tissue.

3. The isolated hepatic rudiment of embryos is at first capable of independent differentiation. In all such grafts the liver appears as an irregularly shaped mass of tubules and sinusoids, histologically normal, but lacking any organization into lobes. An epithelial vesicle identified as the gall bladder occurs in many of the grafts and appears to be normal. Beginning on the twelfth day of development, a degeneration of the hepatic cells of the graft sets in, which by 15 days has become so extensive that many of the hepatic cells are completely disintegrated and there is an infiltration of granulocytes throughout the tissue of the graft.

4. Pancreatic rudiments from donors of approximately 3 days and older have the capacity to undergo self-differentiation within certain limits. (*a*) The Islands of Langerhans, identified by their characteristic alpha and beta cells, appear in abundance throughout the grafts

from donors up to 9 days' incubation. Grafts made from older donors show progressively fewer numbers of islands until in those obtained from 15-day donors there is none. (*b*) The acinar-like tissue appears normal, but when stained selectively for zymogen granules, it always gives negative results, indicating that there is probably no exocrine secretion in the grafted tissue.

5. Paralleling the degenerative processes noted above is an increase of granulocytes, the significance of which is not known.

6. The cessation of growth and subsequent degeneration of liver and pancreatic tissues in grafts to the chorio-allantoic membranes of host embryos apparently coincides with the beginning of functional activity. Their inability to function in the graft leads to their degeneration.

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A QUANTITATIVE STUDY OF RESPIRATORY METABOLISM IN SINGLE DEVELOPING EGGS (ORTHOPTERA)

(Nine figures)

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MANY investigators have recognized insects as favorable material for the study of metabolism. The rates of gaseous exchange during development have been determined for most of the complex stages of the various life-cycles. The feeding larval, the prepupal and pupal stages with their disintegration and rebuilding of tissue, together with the state of quiescence, have been considered by Krogh (1916), Fink (1925), and others.

However, little seems to have been reported, from a quantitative standpoint, upon the respiration of the embryo throughout its entire course of development, and then only in large numbers by such workers as Luciani and Luciani and Piutti (1888; reviewed by Needham, 1931), Melvin (1928), Ashbel (1932), and others. Bodine (1929) observed the phenomenon of diapause in the grasshopper egg and presented quantitatively the rates of respiratory metabolism during the complete development of the eggs when subjected to various degrees of temperature. He demonstrated a rhythm in the rates of oxygen consumption as well as showed that the eggs of grasshoppers may be grouped into three classes depending upon the temperature during which embryonic development normally takes place. In the first class (the one of interest in this paper), the large majority of eggs are known as "diapause type" and must be exposed for a sufficient time to low temperature in order to break the diapause block and thus permit immediate development when returned to a high temperature. The others of this first class, termed "non-diapause type," may continue their development, with little or no cessation of activity, if kept during their entire existence at a constant temperature (28° C.). The eggs of *Melanoplus differentialis*

represent both types and offer splendid opportunity for the study of metabolism during those particular phases of development.

Since previous investigations have given only average rates of oxygen consumption for large numbers of eggs at one time, it was thought advisable to make a study of the oxygen consumption as correlated with the morphological development in the individual grasshopper embryo. This paper contains data on the rates of oxygen consumption during all of the morphological periods through which these two types of eggs pass. These data indicate that, normally, for all eggs of the species (*Melanoplus differentialis*), the percentage of total oxygen consumed during corresponding stages approaches a constant value, irrespective of the length of the period, the amount of oxygen consumed during the period, or the differences in total amount used. When a period varies, either in duration or in the volume of oxygen consumed, from the average of diapause eggs, the period following compensates for the difference.[1]

MATERIAL AND METHODS

Owing to the length of time required, observations on the complete developmental cycle were limited to 92 individual eggs. Of these, 14 died at the end of an abnormal development, 26 gave normal results during formative and diapause periods, and the remaining 42 did likewise during post-diapause. During early experiments, about 50 eggs died due to mercury poisoning resulting from faulty technique.

The eggs used were those laid by wild females, collected early in September of the years 1932 and 1933. These grasshoppers were confined in cages and their eggs collected each day. Eggs were hatched at intervals, and the resulting females, in turn, furnished eggs for part of the later work. Subjected to the same tests, these second generations of eggs were in general indistinguishable, as to the oxygen-consumption rates, from the eggs of the original females.

Experiments were conducted with eggs varying as to the types of treatment received. One group was placed out of doors immediately upon deposition (October), but was removed to the refrigerator

[1] The author wishes to express his appreciation to Professor J. H. Bodine for suggesting the problem, for his continued interest, and just criticism at all times. Also he desires to thank Dr. Eleanor H. Slifer and Dr. Gordon Marsh for advice and help rendered during the entire course of the work.

(2° C.) in February. A second group was kept at room temperature (about 23° C.) for 21 days; at this time some of the eggs were placed out of doors (below developmental zero) and the rest were placed in the refrigerator (2° C.).

Subsequent to the twenty-first day, the chorion or outside shell was removed from all post-diapause and most diapause eggs. This was accomplished by allowing the eggs to dry for a few minutes until the shell or chorion cracked; then gentle rolling of the egg between the fingers removed the chorion. This method for the preparation of the egg, as described by Slifer in 1932, permits splendid visibility of the embryo within the chitinous membrane and allows one to correlate growth with respiratory data.

Because of the exceedingly small amount of oxygen used by the individual egg, a very sensitive measuring apparatus was necessary. The compensating type of manometer (a modification of the Thunberg-Winterstein microrespirometer) designed on the same general plan as Fenn's (1927), but further modified and decreased in size by the author, proved adequate. The diameter of the capillary tube was decreased to $\frac{3}{8}$ mm. and the volume of the flasks to 5 cc. The bottoms of the flasks were elevated conically to permit of a double surface exposure to the water of the constant temperature bath (Fig. 1) and to insure a more rapid attainment of equilibrium between the KOH and the gases in the respective vessels.

During the earlier experiments, 2 eggs were alternately run, in each manometer, for 12 hours. This method was soon discarded and each egg was then measured during its entire development, with the exception of about 2 hours each day, when the manometers were cleaned and the KOH changed. This period of aeration was quite sufficient since, as shown by recent unpublished work of Bodine (personal communication), oxygen tensions from 160 to 20 mm. have no effect upon the respiratory rate of the eggs.

Before experimental work was begun, all manometers were calibrated with mercury, later recalibrated and checked to insure accuracy and the volume computed according to the formula given by Fenn (1927). By multiplying the conversion factor, thus found, by the linear reading upon the manometer, a figure was obtained in terms of gas volume used, per hour, in cubic centimeters.

It is of interest to point out that, after calibrating the manometers with mercury, all embryos kept in them died. This retention of poison by the manometers lasted for several weeks, only gradually wearing away with repeated washings in cleaning fluid after which

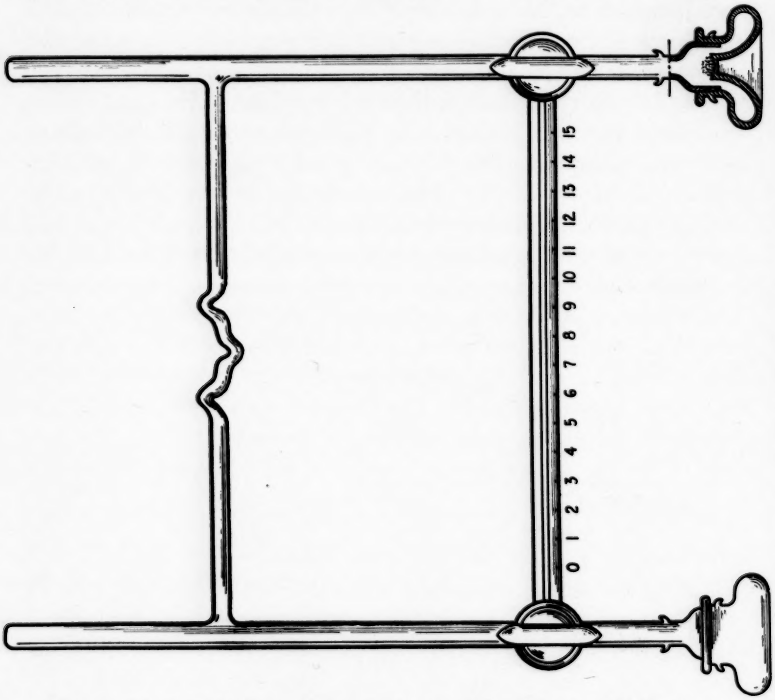

FIG. 1.—Diagram of differential manometer. Special modification of flasks shown at right.

the instrument could be used with safety. For the purpose of a recheck, already mentioned, the manometers were again calibrated. The same mortality occurred. To make certain that the mercury was the cause of the death of the embryos, clean manometers, in which eggs had previously hatched, were treated with mercury. Irrespective of the age or stage of the embryo, its death occurred within 2 days after subjection to the contaminated manometer. Further

tests were made with small vessels which had been washed with mercury. Almost all of the eggs contained in these vessels died, while eggs in control vessels remained unharmed. It is evident that there must be some toxic effect due to the retention of some of the mercury or of mercury compound on the glass. Krogh (1906) suggests the possibility of mercury poisoning, saying, "The introduction of mercury into small animal chamber is also to be deprecated, even if it has not been directly demonstrated that mercury vapor is harmful to the invertebrate animal in question." Krogh does not mention the retention of any mercury by the glass. Possibly the reason why other workers have not experienced this trouble is that the animal chambers of their manometers were not of such small dimensions as those used in the present experiments.

In order to check the accuracy of oxygen-consumption measurements, the same egg (in diapause) was run in each manometer, in turn, for a period of 10 hours. Although there was considerable variation in the readings from different manometers for the same egg (0.00003–0.00025 cc. per hour), this variation was no greater and often less than the variations found when the same egg was measured in one manometer over a series of days (Fig. 7).

All experiments were carried on in a water bath at a constant temperature of 28° ±0.5° C.

During the course of this work the physiological data have been correlated with the morphological changes taking place within the embryo. Nelsen (1931), in his work upon *Melanoplus differentialis*, divides the morphological development of the embryo, exclusive of diapause, into four stages. These periods, together with that of diapause, have been accepted by the author as criteria with which to correlate the measurements of oxygen consumption and are described as follows and illustrated in Figure 2:

A. The pre-revolution period, in which the rudiments of organs and systems are formed [Fig. 2, *Ia* and *Ib*]
B. Diapause, an indefinite period of quiescence [Fig. 2, *Ic*]
C. Post-diapause, divided into the following periods:
   1. The early revolution period, during which the direction of the embryo in the egg is reversed [Fig. 2, *IIa*, *IIb*, and *IIc*]

2. The late revolution period, or time of yolk circumcrescence and completion of the dorsal wall of the embryo [Fig. 2, *III*]
3. The post-revolution period, comprising development from yolk engulfment to hatching [Fig. 2, *IV*]

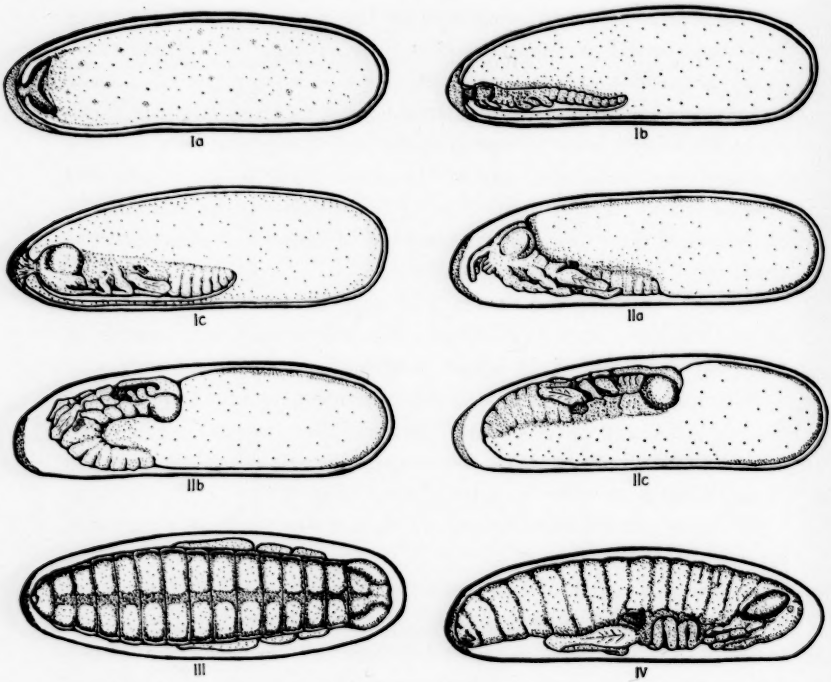

FIG. 2.—Explanation of figures: *Ia*, appearance of germ band, on or near the fifth day; *Ib*, tenth day (average time when oxygen consumption reaches a peak); *Ic*, embryo in diapause (lies on ventral surface with head at the micropyle end of egg); *IIa*, embryo beginning to revolve (the amnio-serosal membranes have been ruptured); *IIb*, embryo is half-revolved; *IIc*, early revolution completed (lateral rotation has not yet taken place); *III*, end of late revolution (dorsal line fused; head at anterior end of egg); *IV*, post-revolution completed and embryo ready to emerge (lying upon ventral side of egg).

The embryological changes have been considered in detail in studies by Nelsen (1931) and Slifer (1930, 1931, 1932), and their results have been used extensively during the course of this work in attempting to tie together morphological and physiological changes.

EXPERIMENTAL RESULTS

I. DIAPAUSE TYPE OF EGG, DEVELOPMENT COMPLETED AFTER SUBJECTION TO 2° C.

A. *Pre-revolution* (Fig. 2, *Ia*, *Ib*).—The amounts of oxygen consumed daily by different eggs (at 28° C.) from deposition until the twenty-first day have been obtained from investigations carried on during the years 1932 and 1933. From these data hourly rates

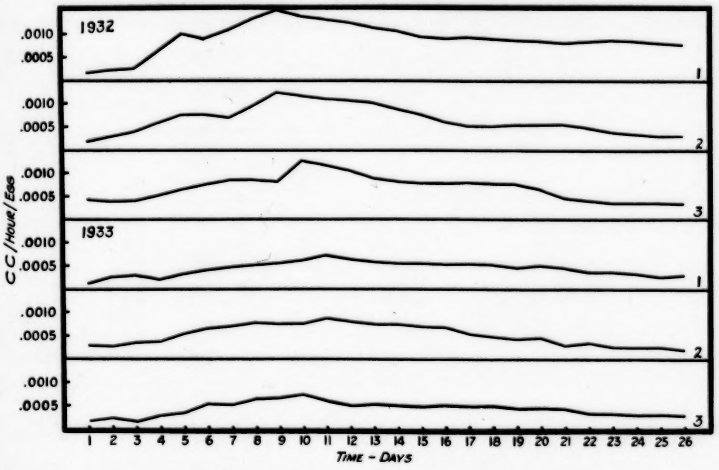

FIG. 3.—Rates of oxygen consumption of typical eggs in diapause, at 28° C. Upper eggs, 1, 2, and 3 for 1932. Lower eggs 1, 2, and 3 for 1933. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated.

(Fig. 3) and the volumes of oxygen consumption during certain periods of days (Table I) have been determined.

The variation in the amount of oxygen consumed by different eggs during the first day may indicate a wide range in the progress of morphological development. In certain eggs only a negligible amount is used during the first 10 hours and little more is consumed during the remainder of the day, while in others the amount is significant from the beginning. The eggs varied in the oxygen consumption, during the first day (Table I, 1932), from 0.00295 to 0.00816 cc. and (1933) from 0.00182 to 0.00540 cc. The average percentages in

terms of total volumes for the two years are close, being 1.3 and 1.6 per cent respectively.

During the first 5 days the rise in the rate of oxygen consumption, in the large majority of eggs, is very gradual (Fig. 3). The values for

TABLE I

AMOUNT OF OXYGEN USED BY TYPICAL EGGS (ACCORDING TO PERIODS) DURING PRE-REVOLUTION; EGG NOS. (BELOW) 1, 2, 3 OF 1932 AND 1, 2, 3 OF 1933 CORRESPOND WITH LIKE NUMBERS OF CURVES IN FIGURE 3

Egg No.	Cc./Oxygen Used during First Day	Per Cent of Total O_2 First Day	Cc./Oxygen Used during First 5 Days	Per Cent of Total O_2 5 Days	Cc./Oxygen for Last 16 Days	Per Cent of Total O_2 16 Days	Cc./Oxygen Used in 21 Days
	1932						
1.....	0.00295	0.7	0.0534	13.8	0.3305	86.0	0.3839
2.....	0.00348	1.2	0.0462	16.3	0.2347	83.5	0.2810
3.....	0.00648	2.0	0.0444	14.2	0.2668	85.7	0.3112
4.....	0.00456	1.2	0.0492	13.3	0.3204	86.6	0.3696
5.....	0.00816	2.3	0.0609	17.8	0.2812	82.1	0.3421
6.....	0.00312	0.8	0.0494	14.4	0.2997	85.8	0.3492
	0.00479	1.3	0.0506	14.9	0.2892	84.9	0.3395
	1933						
1.....	0.00352	1.8	0.0246	13.0	0.1648	86.9	0.1895
2.....	0.00266	1.6	0.0268	17.0	0.1309	82.9	0.1577
3.....	0.00362	2.0	0.0277	15.5	0.1506	84.4	0.1784
4.....	0.00540	2.3	0.0343	15.0	0.1945	84.9	0.2289
5.....	0.00182	1.0	0.0228	12.6	0.1588	87.4	0.1817
112.....	0.00264	1.2	0.0355	16.3	0.1776	83.7	0.2122
	0.00328	1.6	0.0286	14.9	0.1629	85.0	0.1914

oxygen consumed during the 5-day period by different eggs (Table I, 1932) lie between 0.0444 and 0.0609 cc. and (1933) 0.0228 and 0.0355 cc., respectively. The percentage values in terms of total amount of oxygen consumed, for this period, are very close and the averages of the percentages for the two years are constant (Table I). During this time the blastoderm is being laid down and the germinal disk is forming at the caudal end of the egg. The fifth day (28° C.)

usually marks the appearance and invagination of the germ band (Fig. 2, *Ia*). It is at first a small disk of cells comprising the cephalic part of the developing embryo. By approximately the tenth day, the abdomino-thoracic region has tripled in length and segmentation of the abdomen has begun (Fig. 2, *Ib*). (Slifer, 1932, has found this corresponding stage of development to occur at 14 days at 25° C.) This tenth day (average) marks the peak in the rate of oxygen consumption (Fig. 3) during pre-revolution development. (The author has found this point to be retarded to the fourteenth day [average] at 25° C.) From this high point there is a gradual fall until diapause is reached. This decrease in oxygen consumption might not be ex-

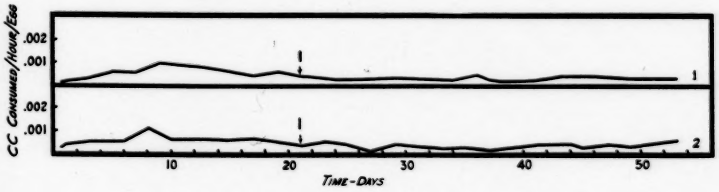

FIG. 4.—Rates of oxygen consumption for 2 typical eggs during pre-revolution and diapause. Diapause begins on the twenty-first day (*Ia*). Ordinates, cubic centimeters per hour per egg. Abscissae, time in days indicated. For further description see text.

pected, inasmuch as in the next 10 days there is a great amount of growth and differentiation, during which time mitotic division is rapidly taking place (Slifer, 1931). Thus it is evident that cell division is not necessarily proportional at all times to the amount of oxygen consumed.

The volumes of oxygen consumed by the different eggs during the last 16 days, as well as that consumed during the first 5 days, as shown above, show a considerable variation. However, a similarity in the percentage values (in terms of total volume) exists. When the averages of the percentages of the years 1932 and 1933 are compared, they are found to be constant.

B. *Diapause.*—During diapause the embryo lies passive at the micropyle end of the egg (Fig. 2, *Ic*). In Figure 4 are shown graphically the rates per hour of two typical eggs from laying until the thirty-second day after the beginning of diapause (*Ia*), or 53 days

after deposition. Their oxygen-consumption rates tend to fall gradually until a period of low usage is reached, on or about the second week (generally) in diapause. (Slifer, 1931, found that mitotic division stops at one week after the beginning of diapause, at 25° C.) This period of low activity continues until about 10 days prior to the breaking of the diapause block. Since eggs Nos. 1 and 2 (Table II, 1932) used the higher volumes during early diapause, and did not progress far into the period of low oxygen consumption, they show a greater hourly average than does egg No. 3 with a long low oxygen consumption. There is considerable fluctuation in the rates of oxygen consumption during diapause. Egg No. 1 varied from

TABLE II

VOLUMES OF OXYGEN CONSUMED BY FOUR TYPICAL EGGS DURING THE FIRST 21 DAYS, DURING DIAPAUSE, THE AVERAGE RATE PER HOUR DURING DIAPAUSE AND TOTAL CONSUMPTION

Egg No.	Volume in Cc. Consumed in Pre-revolution	Number of Days in Diapause	Volume Cc. Consumed in Diapause (*Ia*)	Average Rate/Hour during Diapause	Total Oxygen Used during Entire Time (Cc.)
1	0.2810	32	0.2045	0.000266	0.4855
2	0.3206	32	0.2527	0.000326	0.5734
3	0.2233	91	0.4667	0.000213	0.6900
112	0.2122	37	0.1983	0.000223	0.4106

0.000186 (thirty-seventh day) to 0.000352 cc. (forty-third day); Egg No. 2, from 0.00007 (twenty-seventh day) to 0.00059 cc. (forty-ninth day); and Egg No. 3 fluctuated from 0.000042 (thirty-fourth day) to 0.00024 cc. (forty-sixth day). The cause of this fluctuation in oxygen consumption, during a period when there is no body movement and when the metabolism is presumedly basal, is not known. It might be due to influences exerted within the embryo as it is entering diapause, or when it is preparing to break from the diapause block. This latter phenomenon is shown in Egg No. 3 (Fig. 7) and Egg No. 112 (Fig. 8) during the last 10 days, when the rates rise suddenly in preparation for the beginning of early revolution.

C. *Post-diapause development.*—The different morphological periods, from the end of diapause until hatching (Fig. 2, *IIa–IV*), have been studied and the volumes of oxygen consumed by 42 eggs in

each period of development are shown in Table III. Figures 5 and 6 show curves for oxygen-consumption rates of 8 representative eggs during the periods from diapause to hatching (post-diapause), at a

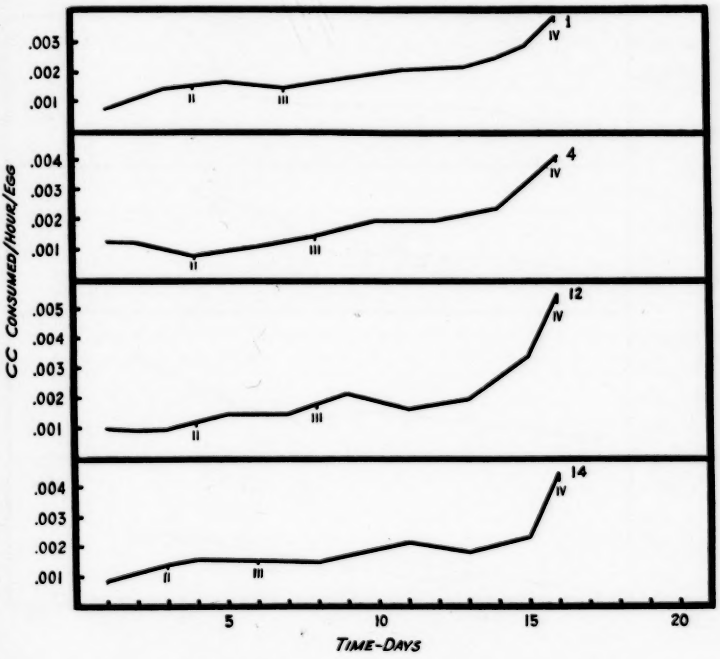

FIG. 5.—Oxygen-consumption rates for 4 typical eggs during post-diapause development at 28° C. (1, 4, 12, and 14 as considered in Table III). Time required for hatching is 16 days, average for all 1932 eggs. Roman numerals designate the end of each developmental period. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated.

constant temperature of 28° C. These eggs, previously subjected to temperature below developmental zero for several months, began to revolve immediately upon being returned to 28° C. The first day shows, in all cases, a rapid rise in the rate of oxygen usage. After this the rate fluctuates slightly, but always recovers quickly and ends with an abrupt ascent just previous to hatching.

Figure 5 shows curves representing the consumption rates of 4

eggs developing very close to the average for typical eggs. They completed their development in the average 16 days. These eggs consumed a very similar amount of oxygen (varying from 0.7238 to

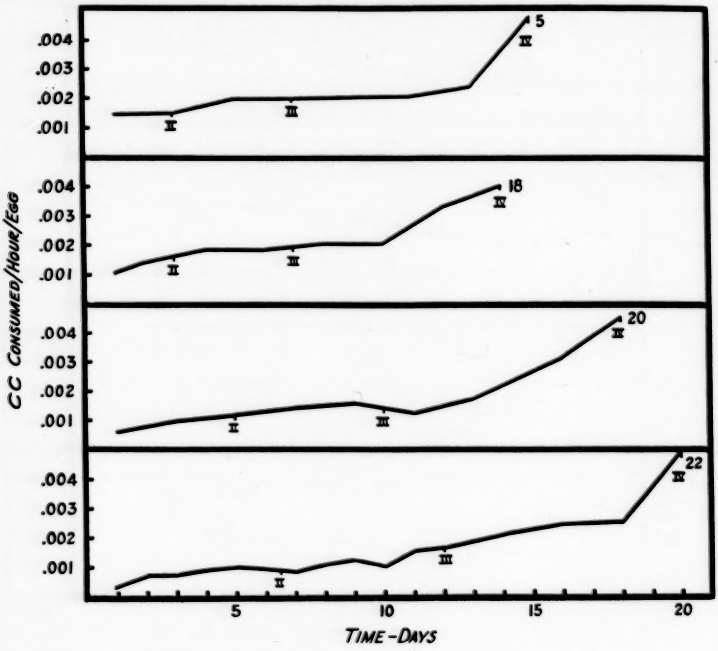

FIG. 6.—Curves represent 4 eggs during post-revolution, showing extreme variations in rates of oxygen consumption and developmental time required at 28° C. Eggs No. 5 and 18 hatched in less than mean time, while Nos. 20 and 22 required 18 and 20 days, respectively. Roman numerals designate the end of each developmental period. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated. For further description see text.

0.7783 cc.) and presented curves of a very similar type. In Figure 6 are shown curves representing extremes in the development of eggs as to their rates of oxygen intake and the length of developmental time. Egg No. 5, showing the highest rates, required the shortest length of time, and the opposite condition is shown in Egg No. 22.

In Table III are given data for the oxygen consumption of eggs

TABLE III

AMOUNT OF OXYGEN USED BY TYPICAL EGGS (ACCORDING TO PERIODS) DURING POST-DIAPAUSE

Egg No.	1. Early Revolution			2. Late Revolution			3. Post-Revolution			Total Days	Total Cc. Used from Diapause to Hatching
	Days in Early Revolution, Period 1	Cc. of O_2 Used during Period 1	Per Cent of Period 1 in Terms of Total Volume	Days in Late Revolution, Period 2	Cc. of O_2 Used during Period 2	Per Cent of Period 2 in Terms of Total Volume	Days in Post-revolution, Period 3	Cc. of O_2 Used during Period 3	Per Cent of Period 3 in Terms of Total Volume		
	1932										
1...	4	0.1056	14.1	3	0.1279	17.1	9	0.5092	68.5	16	0.7428
2...	5	0.1054	19.1	4	0.1127	20.5	9	0.3304	60.1	18	0.5496
3...	5	0.0821	15.9	4	0.1067	20.6	8	0.3260	63.4	17	0.5148
4...	4	0.0982	13.5	4	0.1252	17.2	8	0.5004	69.1	16	0.7238
5...	3	0.1060	12.6	4	0.1900	22.7	8	0.5409	64.5	15	0.8371
6...	3	0.1005	10.0	4	0.2040	24.5	9	0.5277	63.3	16	0.8323
7...	3	0.1039	12.5	5	0.1759	21.3	9	0.5409	65.8	17	0.8208
8...	4	0.1179	13.3	4	0.1852	21.0	9	0.5745	65.4	17	0.8774
9...	3	0.1254	12.5	4	0.2072	20.8	8	0.6622	66.5	15	0.9949
10...	3	0.1555	15.4	4	0.2232	22.3	10	0.6285	62.3	17	1.0089
11...	4	0.1724	17.4	4	0.2124	21.5	9	0.6014	60.9	17	0.9862
12...	4	0.1041	13.3	4	0.1576	20.1	8	0.5164	66.3	16	0.7783
13...	4	0.1771	18.1	4	0.1960	20.0	9	0.6040	61.9	17	0.9772
14...	3	0.1101	15.2	3	0.1455	20.1	10	0.4684	64.7	16	0.7240
15...	3	0.1075	11.9	4	0.1780	19.9	11	0.6055	67.9	18	0.8911
16...	5	0.1669	16.6	3	0.1977	19.7	9	0.6319	63.3	17	0.9966
17...	5	0.1171	16.9	5	0.1444	20.9	9	0.4262	62.0	19	0.6878
18...	3	0.1054	13.3	4	0.1818	22.9	7	0.5007	63.4	14	0.7880
19...	5	0.1724	15.6	3	0.2589	23.4	7	0.6679	60.7	15	1.0992
20...	5	0.1178	13.8	5	0.1735	20.4	8	0.5553	65.6	18	0.8467
21...	4	0.1717	16.3	4	0.2368	22.6	9	0.6374	60.8	17	1.0460
22...	6.5	0.1119	12.8	5.5	0.1684	19.4	8	0.5817	67.4	20	0.8621
23...	3	0.1116	09.6	5	0.2757	23.9	9	0.7622	66.3	17	1.1496
24...	4	0.0958	14.0	4	0.1203	17.6	9	0.4651	68.2	17	0.6813
25...	3	0.1176	14.7	3	0.1667	20.9	9	0.5144	64.4	15	0.7988
26...	4	0.1300	14.7	5	0.1863	21.1	9	0.5659	64.1	18	0.8822
27...	3.5	0.1115	14.9	4.5	0.1542	20.6	8	0.4821	64.4	16	0.7479
28...	4	0.1110	14.4	4	0.1700	22.1	8	0.4880	63.4	16	0.7690
Aver.	4	0.1240±0.003	14.6±0.38	4	0.1779±0.0005	20.8±0.2	8.6	0.5434±0.01	64.4±0.31	16.6	0.8433±0.02

TABLE III—*Continued*

Egg No.	1. Early Revolution			2. Late Revolution			3. Post-Revolution			Total Days	Total Cc. Used from Diapause to Hatching
	Days in Early Revolution, Period 1	Cc. of O_2 Used during Period 1	Per Cent of Period 1 in Terms of Total Volume	Days in Late Revolution, Period 2	Cc. of O_2 Used during Period 2	Per Cent of Period 2 in Terms of Total Volume	Days in Post-revolution, Period 3	Cc. of O_2 Used during Period 3	Per Cent of Period 3 in Terms of Total Volume		
	1933										
1...	3.5	0.0643	11.7	4.5	0.1062	19.7	10	0.3765	66.8	18	0.5471
2...	4	0.1093	15.3	3	0.1185	16.5	8.5	0.4829	67.9	15	0.7109
3...	3.5	0.1111	14.7	3.5	0.1349	17.9	10	0.5063	67.2	17	0.7524
4...	4	0.0944	14.5	3	0.1148	17.7	9	0.4416	67.8	16	0.6512
5...	3	0.1054	14.5	4	0.1412	19.3	9	0.4808	66.0	15	0.7275
6...	3.5	0.0852	15.2	4	0.1054	18.9	10.5	0.2662	65.7	18	0.5571
7...	3	0.0573	11.9	4	0.0935	19.5	11	0.3280	68.4	18	0.4788
8...	3	0.0673	11.8	4	0.1058	18.6	8	0.3952	69.5	15	0.5684
9...	3	0.0861	12.8	4	0.1422	21.2	8	0.4523	67.4	15	0.6708
10...	4	0.0737	13.0	4	0.1301	22.9	8	0.3624	64.0	16	0.5661
11...	3	0.0787	11.5	4	0.1536	22.5	8	0.4492	65.9	16	0.6812
12...	3.5	0.0695	13.9	4	0.0926	18.5	8.5	0.3365	67.4	16	0.4986
13...	4	0.0799	14.2	4	0.1048	18.6	10	0.3703	67.2	19	0.5611
14...	1	0.0793	11.6	4	0.1312	19.4	9	0.4658	68.8	14	0.6764
Aver.	3.3	0.0829±0.003	13.3±0.37	3.8	0.1196±0.0004	19.3±0.4	9	0.4085±0.01	67.1±0.36	16	0.6176±0.02

for the three morphological periods of post-diapause. The average length of time for all periods is shown, as well as the volumes of oxygen consumed and the percentage values in terms of total consumption. The volumes used during the year 1932 are much larger than those used by the eggs measured in 1933, even though experimental conditions were kept uniform. The percentage values representing the corresponding periods of development during both years, however, are constant.

1. *Early revolution* (Fig. 2, *IIa–IIc*).—The type of curve obtained for eggs (representing the rates of oxygen consumption) during early revolution apparently depends upon the thoroughness with which the diapause block has been broken. Eggs which are kept at developmental temperature (28° C.) for a sufficient length of time to permit the proper amount of development of the embryo at the time of entering diapause (3 weeks), and which are then subjected to cold sufficiently long enough to completely break diapause, will revolve in a normal manner. Owing to the fact that early revolution is the most critical process in embryonic development, shortening of the foregoing period of treatment often tends to produce abnormalities as noted below.

When returned to 28° C. from a temperature of 2° C., about $1\frac{1}{2}$ days are required, in average eggs, for the embryo to become active. Waves of contraction may then be seen running along the lateral walls toward the head. This action becomes more intense as the embryo progresses around the micropyle end (Slifer, 1932). The average time required to revolve at 28° C. is 4 days in 1932 and 3.3 days in 1933 (Table III).

In Figure 5 (segment of curve ending with *II*) are given curves representing oxygen consumption of 4 eggs during their early revolution. All of these eggs required very nearly the same amount of oxygen during the period, varying only from 0.0982 to 0.1101 cc. The percentage value for the period varies from 13.3 to 15.2 per cent, which is very close to the average percentage value of 14.6 per cent (Table III, 1932). Figure 6 (segment ending with *II*) shows curves representing eggs which required a wide range of from 3 to $6\frac{1}{2}$ days to complete the period. The oxygen consumed varied in the 4 eggs from 0.1050 to 0.1178 cc. The percentage values were very

close, being from 12.6 to 13.8 per cent. The volume of oxygen consumed for all eggs during the period (Table III) varied from 0.0573 to 0.1771 cc.; yet the percentage values approach a constant.

Eggs kept in the refrigerator (2° C.), from the twenty-first day until placed in the manometer present a higher and more constant percentage value for oxygen consumed during early revolution than do those kept out of doors. Considerable development may take place outside on warm days, even during the winter months (outdoor eggs were removed to the refrigerator in February), and for this reason the percentage value for certain eggs during blastokinesis (Table III, Period 1) may be very low and may not present a true value for the oxygen consumed.

In order to learn the effect upon an embryo of a shortened prerevolution period and a shortened time of subjection to cold, certain eggs were removed from developmental temperature to the refrigerator (2° C.) upon the twelfth day, and kept there for only 8 days longer. They were then placed in the manometer. All of the embryos died but one. This animal was abnormally small and required 35 days to complete blastokinesis. It died on the seventy-third day, being too weak to break out of the chorion. This example perhaps illustrates the results of an improperly broken diapause, which causes an embryo to expend too much time and energy to complete its revolution. The oxygen-consumption rates, as represented by the curve (Fig. 8, *Ia–II*), show the effect upon an egg when it is forced to develop (at a high temperature 28° C.) before the diapause block is entirely eliminated. The result of this was that blastokinesis lasted 24 days and the embryo consumed 0.7574 cc. of oxygen (six times the average for diapause eggs, Table III) in order to overcome the effect of the lingering block. Egg No. 3 (Table II; Fig. 7, *Ia–II*), in spite of the fact that it had not been subjected to cold, developed normally. The embryo was in diapause 91 days, but apparently did not attempt to revolve until the diapause block had been completely eliminated. Post-revolution was normal in all respects.

2. *Late revolution* (Fig. 2, *III*).—The main feature of this period is the growth and extension of the lateral walls in a dorsal direction in order to surround the yolk and to fuse in a dorsal median line.

This fusion is marked, as it begins very early after revolution at the posterior end of the embryo and proceeds forward in a "zipper-like" manner, fastening over the yolk as it progresses until all of the latter, within the serosa, vanishes through the closing head. One can see the ectoderm invaginate to form the proctodeum, and also the mid-gut surround the yolk. The formation of the heart occurs at the end of the period.

Figures 5 and 6 (segment of curve between *II* and *III*) show rates of oxygen consumption, for the same 8 eggs, during late revolution. The length of time required for the completion of development of this period is from 3 to $5\frac{1}{2}$ days (average, 4 days). Table III also shows a large variation in the values of oxygen consumed, but when expressed in terms of percentage volume, all eggs during this period tend to be constant, irrespective of time, rates, or total volumes.

3. *Post-revolution* (Fig. 2, *IV*).—There is a great amount of growth of the embryo taking place during this last period. The engulfed yolk can be seen diminishing as it is being utilized. There is considerable movement of the embryo and the rate of oxygen usage steadily rises. During the last 2 days there is a rapid increase in oxygen requirement, usually ending with an abrupt rise at hatching.

The post-revolution period, as shown in Figures 5 and 6 (segment of curve between *III* and *IV*), varies from 7 to 11 days. The large variations in amounts of oxygen consumed by all eggs, when expressed in terms of percentage of total amount consumed, again show a constant value (see Table III).

IIa. DIAPAUSE TYPE OF EGG, DEVELOPMENT COMPLETED WITHOUT SUBJECTION TO 2° C.

Example 1.—If diapause eggs of *Melanoplus differentialis* are left indefinitely in a high temperature (28° C.), they generally complete their development.

Figure 7 shows a curve representing the rates of oxygen consumption for Egg No. 3 (Table III, 1933), which was never subjected to 2° C. during the 130 days of its development.

The segment of the curve (Fig. 7, *I*) representing the oxygen-consumption rates during pre-revolution agrees favorably with that of all other types of eggs during this period (Fig. 3). The volume of

oxygen consumed is shown in Table III, Egg No. 3, 1933. The oxygen-consumption rates fall steadily from the high point on the ninth day until the thirty-fourth day, or the thirteenth day in diapause. The average rate per hour, during the 91 days in diapause, is 0.000213 cc. (Table II, Egg No. 3). About 14 days prior to the occurrence of revolution, the oxygen consumption begins to rise, indicating the beginning of the breaking of diapause. The chorion was removed from the egg on the one hundred and thirteenth day. This action seemed to have the effect of an impetus in starting blastokinesis, as the embryo started revolution the same day (*Ia*). The remainder of the life-cycle, post-diapause, agrees favorably with the diapause type of egg in regard to oxygen-consumption rates, duration of time, percentage volumes, and total volume of oxygen consumed during corresponding periods (Fig. 7, *II*, *III*, *IV;* Table III, Egg No. 3, 1933).

Example 2.—Figure 8 shows graphically the rates of oxygen consumption of another egg which completed development at a constant temperature of 28° C. in 97 days. The development was abnormal, probably, because the embryo attempted to revolve before the diapause block was entirely eliminated.

The pre-revolution period agrees with all other eggs in respect to both volume of oxygen consumed and rates of consumption per hour (Table I, 1933, Egg No. 112) (Fig. 8, *I*). The peak in the oxygen-consumption rates during pre-revolution is on the eleventh day. The rates slowly drop until the low point in diapause is reached (Fig. 8, *Ia*). Diapause lasted 37 days and is of interest in that there is noted a rise in the oxygen-consumption rate during the last 16 days, doubtless indicating a weakening in the diapause block. Early revolution (Fig. 8, *Ia*, *II*) lasted 24 days as compared with 3.3 days, which is the average time consumed by all eggs during this period (Table III, 1933). There is an intense amount of activity displayed in the form of lateral wall contraction during blastokinesis and the oxygen-consumption rates are higher than at any time during the history of the egg. The volume consumed, 0.7574 cc., is over nine times greater than the average volume of oxygen used by all eggs (where diapause was broken by 2° C.) during like period (Table III, 1933).

FIG. 7.—Rates of oxygen consumed during entire development of a typical egg at 28° C. Roman numerals designate the end of each developmental period. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated.

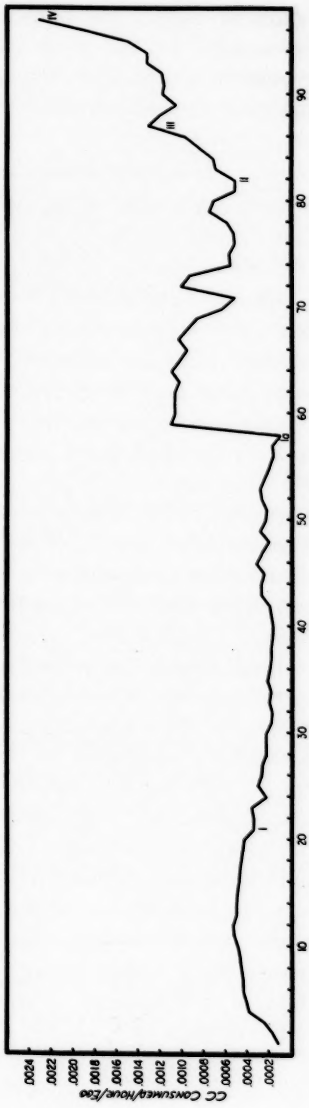

FIG. 8.—Rates of oxygen consumption of a typical egg developing abnormally at 28° C. Roman numerals designate the end of each developmental period. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated.

The rates of oxygen consumption, as well as the volumes of oxygen consumed and the percentage values for both late revolution and post-revolution, agree with figures shown by eggs measured during the year 1933 (Table III).

IIb. DIAPAUSE TYPE OF EGG, SHORTENED DIAPAUSE, DEVELOPMENT COMPLETED WITHOUT SUBJECTION TO 2° C.

In the study made upon 36 eggs during diapause, 5 eggs (normally a very small percentage of diapause eggs show a shortened diapause period) measured showed an unnaturally short diapause. These 5 eggs, not having been subjected to 2° C., but kept from time of deposition at 28° C., remained in diapause for only a few days and then continued their development.

Three of these eggs progressed for only a few days and died. Doubtless the precocious breaking of the diapause block resulted in injury to the embryo. Uvarov (1931), in regard to the phenomenon of the breaking down of the diapause block, says: "Experiments have shown that, when hibernation can be broken by high temperature, a high mortality results, or abnormal individuals are produced. This indicates that heat, instead of merely accelerating the development, acts in this case as a factor disturbing its normal course." Of the remaining 2 eggs, 1 died, possibly due to a too rapid closure of the dorsal wall during late revolution in order to compensate for a too long-continued early revolution.

The history of the 1 egg with an abnormally shortened diapause which successfully completed its development is graphically shown in Figure 9. This egg is presented in order to demonstrate that compensation may exist, in regard to both time and oxygen consumption, in grasshopper eggs. During pre-revolution in this egg, the volume of oxygen consumed is 0.4272 cc. and is 0.0952 cc. greater than the mean consumption of diapause eggs (Table I, 1932). Apparently this greater amount is due to the fact that the breaking of the diapause block began during pre-revolution and permitted a resumption of development. During the diapause period, which lasted only 11½ days (Fig. 9), the oxygen consumed was 0.0639 cc. This is a very small amount when compared to (0.4667 cc.) that consumed by an egg with an uninterrupted diapause (Table II, Egg No. 3).

On the thirty-second day, the embryo began to revolve. The action was sluggish at first but increased with time. Early revolution lasted 13½ days—an extremely long time in comparison with the average time (4 days) for blastokinesis in diapause eggs (Table III, 1932). The amount of oxygen used to accomplish early revolution is 0.1774 cc., or 18 per cent of the total consumption. This figure is slightly larger than those representing oxygen usage in diapause eggs for the period (Table III). However, the fact that blastokinesis was lengthened by 9 days is of interest and undoubtedly indicates a compensation in time for the shortened diapause.

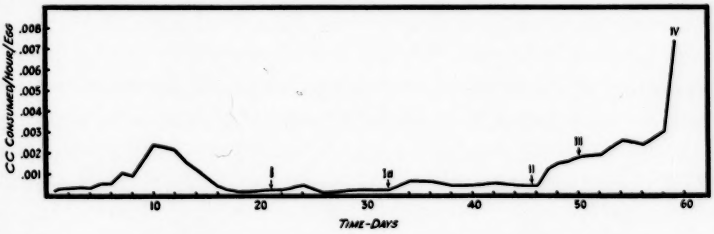

FIG. 9.—Rates of oxygen consumption during the entire development of an egg, with a shortened diapause, at 28° C. Roman numerals designate the end of each developmental period. Ordinates, cubic centimeters of oxygen consumed per hour per egg. Abscissae, time in days indicated. *Ia*, end of formative period, beginning of diapause, 21 days; *Ib*, end of diapause, 11½ days; *II*, end of early revolution, 13½ days; *III*, end of late revolution, 4½ days; *IV*, end of post-revolution, hatching, 9 days.

The last two periods, late revolution and post-revolution, agree in all respects to the average values as given for diapause-type eggs (Table III, 1932). The total amount of oxygen used during the life of this embryo is 1.4738 cc., an amount very close to that of Egg No. 3 (1.4424 cc.), and slightly in excess of the average total volume of all diapause eggs (1.3796 cc.).

DISCUSSION

Bodine and his collaborators have worked out the morphological, physiological, and genetical histories of the eggs of *Melanoplus differentialis*, and the author, using these data as a background, has correlated the oxygen consumption measured during each of the morphological periods in the individual egg.

Bodine (1932) suggests a theory to explain the mechanism of diapause. He shows that the diapause factor is gradually increased in concentration and potency until a threshold value is reached sufficient to suppress gradually the action of the inherent developmental factors. The egg lies dormant until the potency of the block is either eliminated at a low temperature (10°–0° F.) or else becomes gradually weakened at a high temperature (25° C.) to below the threshold value where the developmental factors begin to exercise the greater influence. Normal completion of development depends largely upon the thoroughness with which the diapause block is eliminated. When completely broken at 2° C., the present results show that there exists a constant percentage value (in terms of the total amount of oxygen consumed) for each of the morphological periods for all eggs. This percentage value persists, despite the fact that there is a variation in (*a*) the amount of oxygen consumed during corresponding periods, (*b*) the total amount used during embryonic development, (*c*) the length of the periods, and (*d*) between those eggs measured in 1932 and 1933.

In certain eggs revolution was found to begin before the normal end of diapause. As a result of this action blastokinesis compensated for the shortened time and lessened volume of oxygen used. Egg No. 3 (Fig. 8) serves as an example: It required 24 days to complete the period, instead of the average 3.3 days, and 0.7574 cc. of oxygen, instead of the average amount (0.0829 cc.) consumed by all eggs during revolution (Table III, 1933). The total volumes of oxygen consumed are found to be the same for both the interrupted and the uninterrupted diapause eggs. Similar results were obtained by Krogh (1914) upon the chrysalis of a beetle (*Tenebrio molitor*) when by varying the temperature he was able to change the rates of carbon dioxide output, and found that the length of developmental time varied inversely with the change in rates and that the product of time and rate in all cases gave a constant value. Ashbel (1932) for the silkworm found that the total volume of oxygen consumed by the annual type of eggs was the same as that used during the second generation (diapause) of the bivoltine type. Further, it was shown by Pictet (reviewed by Uvarov, 1932) that in hibernating caterpillars (*Lasiocampa quercus* L.) other factors than temperature cause the

larvae to pupate in the middle of the winter rather than in the spring. However, the pupal stage becomes correspondingly longer, so that the adult emerges at the usual time and the normal cycle is restored. Ludwig (1932), in his work on the Japanese beetle, found the larvae to fall into three groups according to the lengths of their individual instars. In Group I, the first and second instars were shorter than the corresponding instars of Group II, and likewise in Group II they were shorter than in Group III. However, the order was reversed in regard to the length of time consumed during the third instar, and the final result was that all used relatively the same number of days during the total time of the three instars.

SUMMARY AND CONCLUSION

1. Rates of oxygen consumption during each of the morphological periods making up the life-cycle of diapause and certain shortened diapause eggs of *Melanoplus differentialis* (Orthoptera), under constant temperature of 28° C., have been presented.

2. In general, cyclic or rhythmic changes in rates of oxygen consumption of a single developing egg substantiated results obtained upon larger numbers of eggs by previous workers.

3. In the majority of cases it was found that when diapause was broken at a high temperature, death resulted.

4. The percentage volume of oxygen consumed during corresponding periods of development in all eggs approaches a constant, irrespective of their difference in period volumes, their total volume, or time required.

5. In a few cases it is shown that when a period varies from the normal in amount of oxygen consumed or in the time required to complete the particular period, the following period tends to compensate for the difference.

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